



(11)

EP 2 675 464 B1

(12)

EUROPEAN PATENT SPECIFICATION

(45) Date of publication and mention of the grant of the patent:
16.11.2016 Bulletin 2016/46

(51) Int Cl.:
A61K 35/12 (2006.01) C12N 5/073 (2010.01)
C12N 5/079 (2010.01) C12N 5/074 (2010.01)

(21) Application number: 12705594.5

(86) International application number:
PCT/US2012/025086

(22) Date of filing: 14.02.2012

(87) International publication number:
WO 2012/112576 (23.08.2012 Gazette 2012/34)

(54) TREATMENT OF AMYOTROPHIC LATERAL SCLEROSIS USING UMBILICAL DERIVED CELLS

VERWENDUNG VON ZELLEN DER NABELBLUTSCHNUR ZUR BEHANDLUNG DER
AMYOTROPHEN LATERALSKLEROSE

UTILISATION DE CELLULES DÉRIVÉES DU CORDON UMBILICAL POUR LE TRAITEMENT DE
LA SCLÉROSE LATÉRALE AMYOTROPHIQUE

(84) Designated Contracting States:
AL AT BE BG CH CY CZ DE DK EE ES FI FR GB
GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO
PL PT RO RS SE SI SK SM TR

(30) Priority: 14.02.2011 US 201113026995

(43) Date of publication of application:
25.12.2013 Bulletin 2013/52

(83) Declaration under Rule 32(1) EPC (expert solution)

(73) Proprietor: DePuy Synthes Products, Inc.
Raynham, MA 02767 (US)

(72) Inventors:

- GOSIEWSKA, Anna
Skillman, New Jersey 08558 (US)
- HARMON, Alexander M.
Clinton, New Jersey 08890 (US)
- KIHM, Anthony J.
Princeton, New Jersey 08540 (US)
- FANG, Carrie H.
Pittstown, New Jersey 08867 (US)
- DHANARAJ, Sridevi
Raritan, New Jersey 08869 (US)

(74) Representative: Carpmaels & Ransford LLP
One Southampton Row
London WC1B 5HA (GB)

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Remarks:

The file contains technical information submitted after the application was filed and not included in this specification

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Description**FIELD OF THE INVENTION**

5 [0001] This invention relates generally to compositions, methods and kits for cell-based or regenerative therapy for neurological diseases and disorders such as amyotrophic lateral sclerosis. In particular, this invention provides umbilical cord tissue-derived cells for use in preserving motor neuron function in a patient with amyotrophic lateral sclerosis.

BACKGROUND OF THE INVENTION

10 [0002] Various patents and other publications are referred to throughout the specification.
 [0003] Neurological diseases and other disorders of the central and peripheral nervous system are among the most debilitating that can be suffered by an individual, not only because of their physical effects, but also because of their permanence. One of such diseases is amyotrophic lateral sclerosis (ALS) (also known as Lou Gehrig's disease).

15 [0004] ALS is a progressive neurodegenerative disease that mainly affects motor neurons resulting in muscle weakness and atrophy. The disease is characterized by selective, premature degeneration and death of motor neurons. Affected neurons show loss of dendrites, cytoskeletal changes and accumulation of proteins and inclusion bodies. ALS results in a progressive paralysis that is typically fatal within a handful of years due to respiratory failure resulting from paralysis of the respiratory muscles. The average duration of disease from onset to death is three to five years. Only about 10%
 20 of ALS patients survive for ten or more years. As many as 20,000 to 30,000 people in the United States suffer from ALS and an additional 5,000 are diagnosed each year.

25 [0005] The pathogenic mechanisms of ALS are not fully understood but a variety of processes are thought to contribute to ALS including mitochondrial dysfunction, oxidative stress, excitotoxicity as well as alterations in the cytoskeleton, in axonal transport, protein processing, and calcium homeostasis (Ilieva et al. (2009), J. Cell Biol. 187:761-72). Despite intense effort, very limited therapeutic options have emerged for the slowing disease course, although advances have been made in palliative therapy. Allogeneic cell therapy may provide an effective multi-factorial therapy for the treatment of ALS by elaborating trophic factors, which diminish motor neuron degeneration, preserve motor neuron function and extend life.

30 [0006] Given the debilitating effects of ALS and the lack of treatment, there exists a great need for treating ALS in a patient and thereby improving the patient's quality of life.

SUMMARY OF THE INVENTION

35 [0007] The invention provides umbilical cord tissue-derived cells for use in preserving motor neuron function in a patient with amyotrophic lateral sclerosis, wherein the umbilical cord tissue-derived cells are isolatable from human umbilical cord tissue substantially free of blood, are capable of self-renewal and expansion in culture, have the potential to differentiate into cells of other phenotypes, can undergo at least 40 doublings, and have the following characteristics:

40 (a) express each of CD10, CD 13, CD44, CD73, CD90, PDGFr-alpha, PD-L2 and HLA-A,B,C;
 (b) does not express any of CD31, CD34, CD45, CD80, CD86, CD117, CD141, CD 178, B7-H2, HLA-G, or HLA-DR,DP,DQ; and
 (c) have increased expression of interleukin-8; reticulon 1; and chemokine receptor ligand (C-X-C motif) ligand 3, relative to that of a human cell which is a fibroblast, a mesenchymal stem cell, or an iliac crest bone marrow cell.

45 [0008] The problems presented are solved by the compositions, methods and kits of the illustrative embodiments described herein. This invention provides compositions and methods applicable to cell-based or regenerative therapy for neurological diseases and disorders such as amyotrophic lateral sclerosis. In particular, the invention features pharmaceutical compositions, devices and methods for the regeneration or repair of neural tissue using umbilical cord tissue-derived cells.

50 [0009] One aspect of the invention features an isolated umbilical cord tissue-derived cell substantially free of blood, wherein the cell is capable of self-renewal and expansion in culture and has the potential to differentiate into a cell of a neural phenotype; wherein the cell requires L-valine for growth and is capable of growth in at least about 5% oxygen. This cell further comprises one or more of the following characteristics: (a) potential for at least about 40 doublings in culture; (b) attachment and expansion on a coated or uncoated tissue culture vessel, wherein the coated tissue culture vessel comprises a coating of gelatin, laminin, collagen, polyornithine, vitronectin, or fibronectin; (c) production of at least one of tissue factor, vimentin, and alpha-smooth muscle actin; (d) production of at least one of CD10, CD13, CD44, CD73, CD90, PDGFr-alpha, PD-L2 and HLA-A,B,C; (e) lack of production of at least one of CD31, CD34, CD45, CD80, CD86, CD117, CD141, CD178, B7-H2, HLA-G, and HLA-DR,DP,DQ, as detected by flow cytometry; (f) expression of

a gene, which relative to a human cell that is a fibroblast, a mesenchymal stem cell, or an iliac crest bone marrow cell, is increased for at least one of a gene encoding: interleukin 8; reticulon 1; chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha); chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2); chemokine (C-X-C motif) ligand 3; tumor necrosis factor, alpha-induced protein 3; (g) expression of a gene, which relative to a human cell that is a fibroblast, a mesenchymal stem cell, or an iliac crest bone marrow cell, is reduced for at least one of a gene encoding: short stature homeobox 2; heat shock 27 kDa protein 2; chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1); elastin (supravalvular aortic stenosis, Williams-Beuren syndrome); *Homo sapiens* mRNA; cDNA DKFZp586M2022 (from clone DKFZp586M2022); mesenchyme homeo box 2 (growth arrest-specific homeo box); sine oculis homeobox homolog 1 (*Drosophila*); crystallin, alpha B; disheveled associated activator of morphogenesis 2; DKFZP586B2420 protein; similar to neuralin 1; tetranectin (plasminogen binding protein); *src* homology three (SH3) and cysteine rich domain; cholesterol 25-hydroxylase; runt-related transcription factor 3; interleukin 11 receptor, alpha; pro-collagen C-endopeptidase enhancer; frizzled homolog 7 (*Drosophila*); hypothetical gene BC008967; collagen, type VIII, alpha 1; tenascin C (hexabrachion); iroquois homeobox protein 5; hephaestin; integrin, beta 8; synaptic vesicle glycoprotein 2; neuroblastoma, suppression of tumorigenicity 1; insulin-like growth factor binding protein 2, 36kDa; *Homo sapiens* cDNA FLJ12280 fis, clone MAMMA1001744; cytokine receptor-like factor 1; potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4; integrin, beta 7; transcriptional co-activator with PDZ-binding motif (TAZ); sine oculis homeobox homolog 2 (*Drosophila*); KIAA1034 protein; vesicle-associated membrane protein 5 (myobrevin); EGF-containing fibulin-like extracellular matrix protein 1; early growth response 3; distal-less homeo box 5; hypothetical protein FLJ20373; aldo-keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II); biglycan; transcriptional co-activator with PDZ-binding motif (TAZ); fibronectin 1; proenkephalin; integrin, beta-like 1 (with EGF-like repeat domains); *Homo sapiens* mRNA full length insert cDNA clone EUROIMAGE 1968422; EphA3; KIAA0367 protein; natriuretic peptide receptor C/guanylate cyclase C (atrionatriuretic peptide receptor C); hypothetical protein FLJ14054; *Homo sapiens* mRNA; cDNA DKFZp564B222 (from clone DKFZp564B222); BCL2/ad-enovirus E1B 19kDa interacting protein 3-like; AE binding protein 1; cytochrome c oxidase subunit VIIa polypeptide 1 (muscle); similar to neuralin 1; B cell translocation gene 1; hypothetical protein FLJ23191; and DKFZp586L151; (h) secretion of at least one of MCP-1, IL-6, IL-8, GCP-2, HGF, KGF, FGF, HB-EGF, BDNF, TPO, MIP1a, RANTES, and TIMP1; and (i) lack of secretion of at least one of TGF-beta2, ANG2, PDGFbb, MIP1b, I309, MDC, and VEGF, as detected by ELISA.

[0010] In specific embodiments, the umbilicus-derived cell has all identifying features of any one of: cell type UMB 022803 (P7) (ATCC Accession No. PTA-6067); or cell type UMB 022803 (P17) (ATCC Accession No. PTA-6068).

[0011] In certain embodiments, umbilical cord tissue-derived cells are isolated in the presence of one or more enzyme activities comprising metalloprotease activity, mucolytic activity and neutral protease activity. Preferably, the cells have a normal karyotype, which is maintained as the cells are passaged in culture. In preferred embodiments, the postpartum-derived cells comprise each of CD10, CD13, CD44, CD73, CD90, PDGFr-alpha, and HLA-A,B,C and does not comprise any of CD31, CD34, CD45, CD117, CD141, or HLA-DR,DP,DQ, as detected by flow cytometry.

[0012] Another aspect of the invention features a cell population comprising the umbilical cord tissue-derived cells as described above. In one embodiment, the population is a substantially homogeneous population of the umbilical cord tissue-derived cells. In a specific embodiment, the population comprises a clonal cell line of the umbilical cord tissue-derived cells. In another embodiment, the population is a heterogeneous population comprising the umbilical cord tissue-derived cells and at least one other cell type. In certain embodiments, the other cell type is an astrocyte, oligodendrocyte, neuron, neural progenitor, neural stem cell or other multipotent or pluripotent stem cell. In other embodiments, the cell population is cultured in contact with one or more factors that stimulate stem cell differentiation toward a neural lineage.

[0013] Also featured is a cell lysate prepared from umbilical cord tissue-derived cells. The cell lysate may be separated into a membrane enriched fraction and a soluble cell fraction. The disclosure also features an extracellular matrix produced by the umbilical cord tissue-derived cells, as well as a conditioned medium in which the cells have been grown.

[0014] Another aspect features a method of treating a patient having a neurodegenerative condition, the method comprising administering to the patient umbilical cord tissue-derived cells as described above, in an amount effective to treat the neurodegenerative condition. In certain embodiments, the neurodegenerative condition is an acute neurodegenerative condition, such as a brain trauma, spinal cord trauma or peripheral nerve trauma. In other embodiments, it is a chronic or progressive neurodegenerative condition, such as Parkinson's disease, Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis, tumor, multiple sclerosis or chronic peripheral nerve injury.

[0015] In one embodiment, the umbilical cord tissue-derived cells are induced *in vitro* to differentiate into a neural lineage cells prior to administration. In another embodiment, the cells are genetically engineered to produce a gene product that promotes treatment of the neurodegenerative condition.

[0016] In certain embodiments, the cells are administered with at least one other cell type, such as an astrocyte, oligodendrocyte, neuron, neural progenitor, neural stem cell or other multipotent or pluripotent stem cell. In these embodiments, the other cell type can be administered simultaneously with, or before, or after, the umbilical cord tissue-derived cells. Likewise, in these or other embodiments, the cells are administered with at least one other agent, such

as a drug for neural therapy, or another beneficial adjunctive agent such as an anti-inflammatory agent, anti-apoptotic agents, antioxidant or growth factor. In these embodiments, the other agent can be administered simultaneously with, or before, or after, the umbilical cord tissue-derived cells.

[0017] In certain embodiments, the cells are administered at a pre-determined site in the central or peripheral nervous system of the patient. They can be administered by injection or infusion, or encapsulated within an implantable device, or by implantation of a matrix or scaffold containing the cells.

[0018] One embodiment is a method of treating amyotrophic lateral sclerosis comprising administering umbilical cord tissue-derived cells in an amount effective to treat amyotrophic lateral sclerosis to a patient. In this embodiment, the umbilical cord tissue-derived cells are isolated from human umbilical cord tissue substantially free of blood, are capable of self-renewal and expansion into culture, have the potential to differentiate into cells of other phenotypes, can undergo at least 40 doublings, and have the following characteristics: (a) expression each of CD10, CD13, CD44, CD73, CD90, PDGFr-alpha, PD-L2 and HLA-A,B,C; (b) do not express any of CD31, CD34, CD45, CD80, CD86, CD 117, CD141, CD178, B7-H2, HLA-G, or HLA-DR,DP,DQ; and (c) increased expression of interleukin-8; reticulon 1; and chemokine receptor ligand (C-X-C motif) ligand 3, relative to that of a human cell which is a fibroblast, a mesenchymal stem cell, or an iliac crest bone marrow cell. In another embodiment of the method, the umbilical cord tissue-derived cells do not express hTERT or telomerase. In yet another embodiment, the umbilical cord tissue-derived cells are administered by injection (such as e.g. intravenous or intrathecal injection or infusion. In one embodiment, the umbilical cord tissue-derived cells exert a trophic effect on the nervous system of the patient.

[0019] Another embodiment is a method of treating amyotrophic lateral sclerosis comprising administering an effective amount of a substantially homogeneous population of umbilical cord tissue-derived cells to a patient. In this embodiment, the population of umbilical cord tissue-derived cells is isolated from human umbilical cord tissue substantially free of blood, is capable of self-renewal and expansion into culture, has the potential to differentiate into cells of other phenotypes, can undergo at least 40 doublings, and has the following characteristics: (a) expresses each of CD10, CD13, CD44, CD73, CD90, PDGFr-alpha, PD-L2 and HLA-A,B,C; (b) does not express any of CD31, CD34, CD45, CD80, CD86, CD 117, CD141, CD178, B7-H2, HLA-G, or HLA-DR,DP,DQ; and (c) increased expression of interleukin-8; reticulon 1; and chemokine receptor ligand (C-X-C motif) ligand 3, relative to that of a human cell which is a fibroblast, a mesenchymal stem cell, or an iliac crest bone marrow cell. In one embodiment, the umbilical cord tissue-derived cells do not express hTERT or telomerase. In another embodiment, the substantially homogeneous population of umbilical cord tissue-derived cells is administered by injection or infusion. In an alternate embodiment, the substantially homogeneous population of umbilical cord tissue-derived cells is administered by intravenous or intrathecal injection. In yet another embodiment, the substantially homogeneous population of umbilical cord tissue-derived cells exerts a trophic effect on the nervous system of the patient.

[0020] Yet another embodiment is a method of treating amyotrophic lateral sclerosis comprising administering a pharmaceutical composition comprising umbilical cord tissue-derived cells in an amount effective to treat amyotrophic lateral sclerosis to a patient. In this embodiment, the umbilical cord tissue-derived cells are isolated from human umbilical cord tissue substantially free of blood, are capable of self-renewal and expansion into culture, have the potential to differentiate into cells of other phenotypes, can undergo at least 40 doublings, and have the following characteristics: (a) expresses each of CD10, CD13, CD44, CD73, CD90, PDGFr-alpha, PD-L2 and HLA-A,B,C; (b) does not express any of CD31, CD34, CD45, CD80, CD86, CD 117, CD141, CD178, B7-H2, HLA-G, or HLA-DR,DP,DQ; and (c) increased expression of interleukin-8; reticulon 1; and chemokine receptor ligand (C-X-C motif) ligand 3, relative to that of a human cell which is a fibroblast, a mesenchymal stem cell, or an iliac crest bone marrow cell. In one embodiment, the umbilical cord tissue-derived cells do not express hTERT or telomerase. In yet another embodiment, the umbilical cord tissue-derived cells are administered by injection (such as e.g. intravenous or intrathecal injection) or infusion. In one embodiment, the umbilical cord tissue-derived cells exert a trophic effect on the nervous system of the patient.

[0021] Another aspect of the invention features a pharmaceutical composition for treating a patient having a neurodegenerative condition, amyotrophic lateral sclerosis, comprising a pharmaceutically acceptable carrier and the umbilical cord tissue-derived cells described above. The neurodegenerative condition to be treated may be an acute neurodegenerative condition, or it may be a chronic or progressive condition.

[0022] In certain embodiments, the pharmaceutical composition comprises cells that have been induced *in vitro* to differentiate into a neural lineage cells prior to formulation of the composition, or cells that have been genetically engineered to produce a gene product that promotes treatment of the neurodegenerative condition.

[0023] In certain embodiments, the pharmaceutical composition comprises at least one other cell type, such as astrocyte, oligodendrocyte, neuron, neural progenitor, neural stem cell or other multipotent or pluripotent stem cell. In these or other embodiments, the pharmaceutical composition comprises at least one other agent, such as a drug for neural therapy, or another beneficial adjunctive agent such as an anti-inflammatory agent, anti-apoptotic agents, antioxidant or growth factor.

[0024] In certain embodiments, the pharmaceutical composition is formulated for administration by injection or infusion. Alternatively, it may comprise an implantable device in which the cells are encapsulated, or a matrix or scaffold containing

the cells.

[0025] According to yet another aspect of the invention, a kit is provided for treating a patient having a neurodegenerative condition. The kit comprises a pharmaceutically acceptable carrier, a population of the above-described umbilical cord tissue-derived cells and instructions for using the kit in a method of treating the patient. The kit may further comprise at least one reagent and instructions for culturing the umbilical cord tissue-derived cells. It may also comprise a population of at least one other cell type, or at least one other agent for treating a neurodegenerative condition.

[0026] According to another aspect, a method is provided for treating a patient having a neurodegenerative condition (such as e.g. amyotrophic lateral sclerosis), which comprises administering to the patient a preparation made from the above-described umbilical cord tissue-derived cells. Such a preparation may comprise a cell lysate (or fraction thereof) of the umbilical cord tissue-derived cells, an extracellular matrix of the umbilical cord tissue-derived cells, or a conditioned medium in which the umbilical cord tissue-derived cells were grown. Another aspect features a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a preparation made from the umbilical cord tissue-derived cells, which may be a cell lysate (or fraction thereof) of the umbilical cord tissue-derived cells, an extracellular matrix of the umbilical cord tissue-derived cells or a conditioned medium in which the umbilical cord tissue-derived cells were grown. Kits for practicing this aspect of the invention are also provided. These may include the one or more of a pharmaceutically acceptable carrier or other agent or reagent, one or more of a cell lysate or fraction thereof, an extracellular matrix or a conditioned medium from the umbilical cord tissue-derived cells, and instructions for use of the kit components.

[0027] Other features and advantages of the invention will be apparent from the detailed description and examples that follow.

BRIEF DESCRIPTION OF THE DRAWINGS

[0028]

25 **Figure 1** shows the monitoring of animal weight throughout the duration of the study.

Figure 2 shows the response of the different cell groups to amorphine challenge.

Figure 3 shows the monitoring of the difference in the number of head turns of left and right throughout the duration of the study.

30 **Figure 4** shows the monitoring of the consumption of food over by the animals throughout the duration of the study using a staircase challenge.

Figure 5 shows bar graphs illustrating the qualitative assessment of (a) Iba-1; (b) ED-1; and, (C) DAPI staining performed in the cell graft according to the following criteria: 0 = None (absence of cells); 1 = Visible staining; 2 = Abundant staining; 3 = Very abundant staining; 4 = Dense.

35 **Figure 6** shows bar graphs illustrating the qualitative assessment of (a) GFAP and (b) Vimentin staining performed in the cell graft according to the following criteria: 0 = None (absence of cells); 1 = Visible staining; 2 = Abundant staining; 3 = Very abundant staining; 4 = Dense.

Figure 7 shows the Effect of human umbilical cord tissue-derived cells ("hUTC") on Survival in SOD1(G93A) Rat Model of ALS for (A) Groups 1 and 2 and (B) Groups 3 and 4.

40 **Figure 8** shows life span of animals in the groups that were administered the control vehicle and the hUTC (see Example 21). The bar diagram shows that the cell treated groups, especially in Arm 2, have a longer life span by 15.75-day (2.25 wks, P<0.035) than the vehicle groups although the age of disease onset is similar.

Figure 9 shows the locomotion activity of animals treated with or without hUTC. Figure 9A shows the Basso, Beattie and Bresnahan (BBB) test scores for Groups 1 and 2. Figure 9B shows the inclined plane performance test scores for Groups 1 and 2. Figure 9C shows the BBB scores for Groups 3 and 4. Figure 9D shows the inclined plane scores for Groups 3 and 4. The data demonstrates the separation in the two principal measures of muscle weakness in the vehicle and cell treated groups.

Figure 10 shows the survival curve for the 10-week arm (for all subjects).

Figure 11 shows the survival curve for the 12-week arm (for all subjects).

50 **Figure 12** shows the survival curve for the 12-week arm (last two pairs).

Figure 13 shows the inclined plane results for the 10-week arm.

Figure 14 shows the BBB scores for the 10-week arm.

Figure 15 shows the inclined plane results for the 12-week arm for the first six pairs.

Figure 16 shows the inclined plane results for the 12-week arm for the last two pairs.

Figure 17 shows the BBB scores for the 12-week arm for the first six pairs.

55 **Figure 18** shows the BBB scores for the 12-week arm for the last two pairs.

Figure 19 shows the appearance of creysl-violet stained lumbar cord sections from SOD1 G93A rats. Figure 19A shows the appearance of creysl-violet stained lumbar cord sections from odd number animals. Figure 19B shows the appearance of creysl-violet stained lumbar cord sections from even number animals. Figure 19C shows the

appearance of creysl-violet stained lumbar cord sections from normal animals.

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

5 **Definitions**

[0029] Various terms used throughout the specification and claims are defined as set forth below.

[0030] *Stem cells* are undifferentiated cells defined by the ability of a single cell both to self-renew, and to differentiate to produce progeny cells, including self-renewing progenitors, non-renewing progenitors, and terminally differentiated cells. Stem cells are also characterized by their ability to differentiate *in vitro* into functional cells of various cell lineages from multiple germ layers (endoderm, mesoderm and ectoderm), as well as to give rise to tissues of multiple germ layers following transplantation, and to contribute substantially to most, if not all, tissues following injection into blastocysts.

[0031] Stem cells are classified according to their developmental potential as: (1) *totipotent*; (2) *pluripotent*; (3) *multipotent*; (4) *oligopotent*; and (5) *unipotent*. *Totipotent* cells are able to give rise to all embryonic and extraembryonic cell types. *Pluripotent* cells are able to give rise to all embryonic cell types. *Multipotent* cells include those able to give rise to a subset of cell lineages, but all within a particular tissue, organ, or physiological system (for example, hematopoietic stem cells (HSC) can produce progeny that include HSC (self-renewal), blood cell-restricted oligopotent progenitors, and all cell types and elements (e.g., platelets) that are normal components of the blood). Cells that are *oligopotent* can give rise to a more restricted subset of cell lineages than multipotent stem cells; and cells that are *unipotent* are able to give rise to a single cell lineage (e.g., spermatogenic stem cells).

[0032] Stem cells are also categorized on the basis of the source from which they may be obtained. An *adult stem cell* is generally a multipotent undifferentiated cell found in tissue comprising multiple differentiated cell types. The adult stem cell can renew itself. Under normal circumstances, it can also differentiate to yield the specialized cell types of the tissue from which it originated, and possibly other tissue types. An *embryonic stem cell* is a pluripotent cell from the inner cell mass of a blastocyst-stage embryo. A *fetal* stem cell is one that originates from fetal tissues or membranes. A *postpartum stem cell* is a multipotent or pluripotent cell that originates substantially from extraembryonic tissue available after birth, namely, the placenta and the umbilical cord. These cells have been found to possess features characteristic of pluripotent stem cells, including rapid proliferation and the potential for differentiation into many cell lineages. Post-partum stem cells may be blood-derived (e.g., as are those obtained from umbilical cord blood) or non-blood-derived (e.g., as obtained from the non-blood tissues of the umbilical cord and placenta).

[0033] *Embryonic tissue* is typically defined as tissue originating from the embryo (which in humans refers to the period from fertilization to about six weeks of development). *Fetal tissue* refers to tissue originating from the fetus, which in humans refers to the period from about six weeks of development to parturition. *Extraembryonic tissue* is tissue associated with, but not originating from, the embryo or fetus. Extraembryonic tissues include extraembryonic membranes (chorion, amnion, yolk sac and allantois), umbilical cord and placenta (which itself forms from the chorion and the maternal decidua basalis).

[0034] *Differentiation* is the process by which an unspecialized ("uncommitted") or less specialized cell acquires the features of a specialized cell, such as a nerve cell or a muscle cell, for example. A *differentiated* cell is one that has taken on a more specialized ("committed") position within the lineage of a cell. The term *committed*, when applied to the process of differentiation, refers to a cell that has proceeded in the differentiation pathway to a point where, under normal circumstances, it will continue to differentiate into a specific cell type or subset of cell types, and cannot, under normal circumstances, differentiate into a different cell type or revert to a less differentiated cell type. *De-differentiation* refers to the process by which a cell reverts to a less specialized (or committed) position within the lineage of a cell. As used herein, the *lineage* of a cell defines the heredity of the cell, *i.e.* which cells it came from and what cells it can give rise to. The lineage of a cell places the cell within a hereditary scheme of development and differentiation.

[0035] In a broad sense, a *progenitor cell* is a cell that has the capacity to create progeny that are more differentiated than itself, and yet retains the capacity to replenish the pool of progenitors. By that definition, stem cells themselves are also progenitor cells, as are the more immediate precursors to terminally differentiated cells. When referring to the cells of the present invention, as described in greater detail below, this broad definition of *progenitor cell* may be used. In a narrower sense, a progenitor cell is often defined as a cell that is intermediate in the differentiation pathway, *i.e.*, it arises from a stem cell and is intermediate in the production of a mature cell type or subset of cell types. This type of progenitor cell is generally not able to self-renew. Accordingly, if this type of cell is referred to herein, it will be referred to as a *non-renewing progenitor cell* or as an *intermediate progenitor or precursor cell*.

[0036] As used herein, the phrase *differentiates into a neural lineage or phenotype* refers to a cell that becomes partially or fully committed to a specific neural phenotype of the CNS or PNS, *i.e.*, a neuron or a glial cell, the latter category including without limitation astrocytes, oligodendrocytes, Schwann cells and microglia.

[0037] The cells of the present invention are generally referred to as *umbilical cord tissue-derived cells* (or *UTC* or *hUTCs*). They also may sometimes be referred to as *umbilicus-derived cells (UDCs)*. In addition, the cells may be

described as being stem or progenitor cells, the latter term being used in the broad sense. The term *derived* is used to indicate that the cells have been obtained from their biological source and grown or otherwise manipulated *in vitro* (e.g., cultured in a Growth Medium to expand the population and/or to produce a cell line). The *in vitro* manipulations of umbilical stem cells and the unique features of the umbilicus-derived cells of the present invention are described in detail below.

[0038] Various terms are used to describe cells in culture. *Cell culture* refers generally to cells taken from a living organism and grown under controlled condition ("in culture" or "cultured"). A *primary cell culture* is a culture of cells, tissues, or organs taken directly from an organism(s) before the first subculture. Cells are *expanded* in culture when they are placed in a Growth Medium under conditions that facilitate cell growth and/or division, resulting in a larger population of the cells. When cells are expanded in culture, the rate of cell proliferation is sometimes measured by the amount of time needed for the cells to double in number. This is referred to as *doubling time*.

[0039] A *cell line* is a population of cells formed by one or more subcultivations of a primary cell culture. Each round of subculturing is referred to as a *passage*. When cells are subcultured, they are referred to as having been *passaged*. A specific population of cells, or a cell line, is sometimes referred to or characterized by the number of times it has been passaged. For example, a cultured cell population that has been passaged ten times may be referred to as a *P10* culture. The primary culture, *i.e.*, the first culture following the isolation of cells from tissue, is designated *P0*. Following the first subculture, the cells are described as a secondary culture (*P1* or passage 1). After the second subculture, the cells become a tertiary culture (*P2* or passage 2), and so on. It will be understood by those of skill in the art that there may be many population doublings during the period of passaging; therefore, the number of population doublings of a culture is greater than the passage number. The expansion of cells (*i.e.*, the number of population doublings) during the period between passaging depends on many factors, including but not limited to the seeding density, substrate, medium, growth conditions, and time between passaging.

[0040] A *conditioned medium* is a medium in which a specific cell or population of cells has been cultured, and then removed. When cells are cultured in a medium, they may secrete cellular factors that can provide trophic support to other cells. Such trophic factors include, but are not limited to hormones, cytokines, extracellular matrix (ECM), proteins, vesicles, antibodies, and granules. The medium containing the cellular factors is the conditioned medium.

[0041] Generally, a *trophic factor* is defined as a substance that promotes survival, growth, differentiation, proliferation and /or maturation of a cell, or stimulates increased activity of a cell. The interaction between cells *via* trophic factors may occur between cells of different types. Cell interaction by way of trophic factors is found in essentially all cell types, and is a particularly significant means of communication among neural cell types. Trophic factors also can function in an autocrine fashion, *i.e.*, a cell may produce trophic factors that affect its own survival, growth, differentiation, proliferation and/or maturation.

[0042] When referring to cultured vertebrate cells, the term *senescence* (also *replicative senescence* or *cellular senescence*) refers to a property attributable to finite cell cultures; namely, their inability to grow beyond a finite number of population doublings (sometimes referred to as *Hayflick's limit*). Although cellular senescence was first described using fibroblast-like cells, most normal human cell types that can be grown successfully in culture undergo cellular senescence. The *in vitro* lifespan of different cell types varies, but the maximum lifespan is typically fewer than 100 population doublings (this is the number of doublings for all the cells in the culture to become senescent and thus render the culture unable to divide). Senescence does not depend on chronological time, but rather is measured by the number of cell divisions, or population doublings, the culture has undergone. Thus, cells made quiescent by removing essential growth factors are able to resume growth and division when the growth factors are re-introduced, and thereafter carry out the same number of doublings as equivalent cells grown continuously. Similarly, when cells are frozen in liquid nitrogen after various numbers of population doublings and then thawed and cultured, they undergo substantially the same number of doublings as cells maintained unfrozen in culture. Senescent cells are not dead or dying cells; they are actually resistant to programmed cell death (apoptosis), and have been maintained in their nondividing state for as long as three years. These cells are very much alive and metabolically active, but they do not divide. The nondividing state of senescent cells has not yet been found to be reversible by any biological, chemical, or viral agent.

[0043] The term *neurodegenerative condition* (*nor disorder*) is an inclusive term encompassing acute and chronic conditions, disorders or diseases of the central or peripheral nervous system. A neurodegenerative condition may be age-related, or it may result from injury or trauma, or it may be related to a specific disease or disorder. Acute neurodegenerative conditions include, but are not limited to, conditions associated with neuronal cell death or compromise including cerebrovascular insufficiency, focal or diffuse brain trauma, diffuse brain damage, spinal cord injury or peripheral nerve trauma, *e.g.*, resulting from physical or chemical burns, deep cuts or limb severance. Examples of acute neurodegenerative disorders are: cerebral ischemia or infarction including embolic occlusion and thrombotic occlusion, reperfusion following acute ischemia, perinatal hypoxic-ischemic injury, cardiac arrest, as well as intracranial hemorrhage of any type (such as epidural, subdural, subarachnoid and intracerebral), and intracranial and intravertebral lesions (such as contusion, penetration, shear, compression and laceration), as well as whiplash and shaken infant syndrome. Chronic neurodegenerative conditions include, but are not limited to, Alzheimer's disease, Pick's disease, diffuse Lewy body

disease, progressive supranuclear palsy (Steel-Richardson syndrome), multisystem degeneration (Shy-Drager syndrome), chronic epileptic conditions associated with neurodegeneration, motor neuron diseases including amyotrophic lateral sclerosis, degenerative ataxias, cortical basal degeneration, ALS-Parkinson's-Dementia complex of Guam, subacute sclerosing panencephalitis, Huntington's disease, Parkinson's disease, synucleinopathies (including multiple system atrophy), primary progressive aphasia, striatonigral degeneration, Machado-Joseph disease / spinocerebellar ataxia type 3 and olivopontocerebellar degenerations, Gilles De La Tourette's disease, bulbar and pseudobulbar palsy, spinal and spinobulbar muscular atrophy (Kennedy's disease), primary lateral sclerosis, familial spastic paraparesis, Werdnig-Hoffmann disease, Kugelberg-Welander disease, Tay-Sach's disease, Sandhoff disease, familial spastic disease, Wohlfart-Kugelberg-Welander disease, spastic paraparesis, progressive multifocal leukoencephalopathy, familial dysautonomia (Riley-Day syndrome), and prion diseases (including, but not limited to Creutzfeldt-Jakob, Gerstmann-Straussler-Scheinker disease, Kuru and fatal familial insomnia), demyelination diseases and disorders including multiple sclerosis and hereditary diseases such as leukodystrophies.

[0044] Other neurodegenerative conditions include tumors and other neoplastic conditions affecting the CNS and PNS. Though the underlying disease is considered proliferative (rather than neurodegenerative), surrounding tissues may be compromised. Furthermore, cell therapy may be utilized to deliver apoptotic or other antineoplastic molecules to the tumor site, e.g., via delivery of genetically modified cells producing such agents.

[0045] Other neurodegenerative conditions include various neuropathies, such as multifocal neuropathies, sensory neuropathies, motor neuropathies, sensory-motor neuropathies, infection-related neuropathies, autonomic neuropathies, sensory-autonomic neuropathies, demyelinating neuropathies (including, but not limited to, Guillain-Barre syndrome and chronic inflammatory demyelinating polyradiculoneuropathy), other inflammatory and immune neuropathies, neuropathies induced by drugs, neuropathies induced by pharmacological treatments, neuropathies induced by toxins, traumatic neuropathies (including, but not limited to, compression, crush, laceration and segmentation neuropathies), metabolic neuropathies, endocrine and paraneoplastic neuropathies, among others.

[0046] Other neurodegenerative conditions include dementias, regardless of underlying etiology, including age-related dementia and other dementias and conditions with memory loss including dementia associated with Alzheimer's disease, vascular dementia, diffuse white matter disease (Binswanger's disease), dementia of endocrine or metabolic origin, dementia of head trauma and diffuse brain damage, dementia pugilistica and frontal lobe dementia.

[0047] The term *treating (nor treatment of) a neurodegenerative condition* refers to ameliorating the effects of, or delaying, halting or reversing the progress of, or delaying or preventing the onset of, a neurodegenerative condition as defined herein.

[0048] The term *effective amount* refers to a concentration or amount of a reagent or pharmaceutical composition, such as a growth factor, differentiation agent, trophic factor, cell population or other agent, that is effective for producing an intended result, including cell growth and/or differentiation *in vitro* or *in vivo*, or treatment of a neurodegenerative condition as described herein. With respect to growth factors, an effective amount may range from about 1 nanogram/milliliter to about 1 microgram/milliliter. With respect to cells as administered to a patient *in vivo*, an effective amount may range from as few as several hundred or fewer to as many as several million or more. In specific embodiments, an effective amount may range from 10^3 - 10^{11} , more specifically at least about 10^4 cells. It will be appreciated that the number of cells to be administered will vary depending on the specifics of the disorder to be treated, including but not limited to size or total volume/surface area to be treated, as well as proximity of the site of administration to the location of the region to be treated, among other factors familiar to the medicinal biologist.

[0049] The terms *effective period (or time)* and *effective conditions* refer to a period of time or other controllable conditions (e.g., temperature, humidity for *in vitro* methods), necessary or preferred for an agent or pharmaceutical composition to achieve its intended result.

[0050] The term *patient or subject* refers to animals, including mammals, preferably humans, who are treated with the pharmaceutical compositions or in accordance with the methods described herein.

[0051] The term *pharmaceutically acceptable carrier (or Medium)*, which may be used interchangeably with the term *biologically compatible carrier or Medium*, refers to reagents, cells, compounds, materials, compositions, and/or dosage forms that are not only compatible with the cells and other agents to be administered therapeutically, but also are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other complication commensurate with a reasonable benefit/risk ratio. As described in greater detail herein, pharmaceutically acceptable carriers suitable for use in the present invention include liquids, semi-solid (e.g., gels) and solid materials (e.g., cell scaffolds and matrices, tubes sheets and other such materials as known in the art and described in greater detail herein). These semi-solid and solid materials may be designed to resist degradation within the body (*non-biodegradable*) or they may be designed to degrade within the body (*biodegradable, bioerodable*). A biodegradable material may further be *bioresorbable or bioabsorbable*, i.e., it may be dissolved and absorbed into bodily fluids (water-soluble implants are one example), or degraded and ultimately eliminated from the body, either by conversion into other materials or breakdown and elimination through natural pathways.

[0052] Several terms are used herein with respect to cell replacement therapy. The terms *autologous transfer, autol-*

ogous transplantation, autograft and the like refer to treatments wherein the cell donor is also the recipient of the cell replacement therapy. The terms *allogeneic transfer*, *allogeneic transplantation*, *allograft* and the like refer to treatments wherein the cell donor is of the same species as the recipient of the cell replacement therapy, but is not the same individual. A cell transfer in which the donor's cells have been histocompatibly matched with a recipient is sometimes referred to as a *syngeneic transfer*. The terms *xenogeneic transfer*, *xenogeneic transplantation*, *xenograft* and the like refer to treatments wherein the cell donor is of a different species than the recipient of the cell replacement therapy.

Description

[0053] Neurodegenerative conditions, which encompass acute, chronic and progressive disorders and diseases having widely divergent causes, have as a common feature the dysfunction or loss of a specific or vulnerable group of neural cells. This commonality enables development of similar therapeutic approaches for the repair and regeneration of vulnerable or damaged neural tissue, one of which is cell-based therapy. In its various embodiments described herein, the present invention features methods and pharmaceutical compositions for neural repair and regeneration that utilize progenitor cells and cell populations derived from umbilical cord tissue. The disclosure is applicable to any neurodegenerative condition, but is expected to be particularly suitable for a number of neural disorders for which treatment or cure heretofore has been difficult or unavailable. These include, without limitation, Parkinson's disease, Alzheimer's disease, Huntington's disease, stroke, amyotrophic lateral sclerosis, multiple sclerosis, spinal cord injury and peripheral nerve injury (e.g., as associated with diabetic neuropathy). In one embodiment of the invention, the neurodegenerative condition is amyotrophic lateral sclerosis (ALS).

[0054] As summarized above, the invention, in one of its aspects is generally directed to isolated umbilical cord tissue-derived cells (UTCs), which have been rendered substantially free of blood. The UTCs are capable of self-renewal and expansion in culture and have the potential to differentiate into cells of neural phenotypes. Certain embodiments feature populations comprising such cells, pharmaceutical compositions comprising the cells or components or products thereof, and methods of using the pharmaceutical compositions for treatment of patients with acute or chronic neurodegenerative conditions. The umbilical cord tissue-derived cells have been characterized by their growth properties in culture, by their cell surface markers, by their gene expression, by their ability to produce certain biochemical trophic factors, and by their immunological properties.

Preparation of Umbilicus and Placenta Derived Tissue Cells

[0055] According to the methods described herein, a mammalian placenta and umbilical cord are recovered upon or shortly after termination of either a full-term or pre-term pregnancy, for example, after expulsion after birth. The tissue may be transported from the birth site to a laboratory in a sterile container such as a flask, beaker, culture dish, or bag. The container may have a solution or medium, including but not limited to a salt solution, such as, for example, Dulbecco's Modified Eagle's Medium (DMEM) or phosphate buffered saline (PBS), or any solution used for transportation of organs used for transplantation, such as University of Wisconsin solution or perfluorochemical solution. One or more antibiotic and/or antimycotic agents, such as but not limited to penicillin, streptomycin, amphotericin B, gentamicin, and nystatin, may be added to the medium or buffer. The umbilical and placenta tissue may be rinsed with an anticoagulant solution such as heparin-containing solution. It is preferable to keep the tissue at about 4-10°C prior to extraction of the cells. It is even more preferable that the tissue not be frozen prior to extraction of the umbilical or placenta-derived cells.

[0056] Isolation of cells preferably occurs in an aseptic environment. The umbilical cord may be separated from the placenta by means known in the art. Alternatively, the umbilical cord and placenta are used without separation. Blood and debris are preferably removed from the tissue prior to isolation of cells. For example, the tissue may be washed with buffer solution, such as but not limited to phosphate buffered saline. The wash buffer also may comprise one or more antimycotic and/or antibiotic agents, such as but not limited to penicillin, streptomycin, amphotericin B, gentamicin, and nystatin.

[0057] Tissue comprising a whole umbilical cord, whole placenta or a fragment or section thereof is disaggregated by mechanical force (mincing or shear forces). In a presently preferred embodiment, the isolation procedure also utilizes an enzymatic digestion process. Many enzymes are known in the art to be useful for the isolation of individual cells from complex tissue matrices to facilitate growth in culture. Ranging from weakly digestive (e.g. deoxyribonucleases and the neutral protease, dispase) to strongly digestive (e.g. papain and trypsin), such enzymes are available commercially. A nonexhaustive list of enzymes compatible herewith includes mucolytic enzyme activities, metalloproteases, neutral proteases, serine proteases (such as trypsin, chymotrypsin, or elastase), and deoxyribonucleases. Presently preferred are enzyme activities selected from metalloproteases, neutral proteases and mucolytic activities. For example, collagenases are known to be useful for isolating various cells from tissues. Deoxyribonucleases can digest single-stranded DNA and can minimize cell-clumping during isolation. Preferred methods involve enzymatic treatment with for example collagenase and dispase, or collagenase, dispase, and hyaluronidase, and such methods are provided wherein in certain

preferred embodiments, a mixture of collagenase and the neutral protease dispase are used in the dissociating step. More preferred are those methods which employ digestion in the presence of at least one collagenase from *Clostridium histolyticum*, and either of the protease activities, dispase and thermolysin. Still more preferred are methods employing digestion with both collagenase and dispase enzyme activities. Also preferred are methods which include digestion with a hyaluronidase activity in addition to collagenase and dispase activities. The skilled artisan will appreciate that many such enzyme treatments are known in the art for isolating cells from various tissue sources. For example, the LIBERASE™ Blendzyme (Roche) series of enzyme combinations are suitable for use in the instant methods. Other sources of enzymes are known, and the skilled artisan may also obtain such enzymes directly from their natural sources. The skilled artisan is also well-equipped to assess new, or additional enzymes or enzyme combinations for their utility in isolating the cells of the invention. Preferred enzyme treatments are 0.5, 1, 1.5, or 2 hours long or longer. In other preferred embodiments, the tissue is incubated at 37°C during the enzyme treatment of the dissociation step.

[0058] In some embodiments of the invention, the tissue is separated into sections comprising various aspects of the tissue, such as neonatal, neonatal/maternal, and maternal aspects of the placenta, for instance. The separated sections then are dissociated by mechanical and/or enzymatic dissociation according to the methods described herein. Cells of neonatal or maternal lineage may be identified by any means known in the art, for example, by karyotype analysis or *in situ* hybridization for a Y chromosome.

[0059] Isolated cells or tissue from which hUTCs grow out may be used to initiate, or seed, cell cultures. Isolated cells are transferred to sterile tissue culture vessels either uncoated or coated with extracellular matrix or ligands such as laminin, collagen (native, denatured or crosslinked), gelatin, fibronectin, and other extracellular matrix proteins. hUTCs are cultured in any culture medium capable of sustaining growth of the cells such as, but not limited to, DMEM (high or low glucose), advanced DMEM, DMEM/MCDB 201, Eagle's basal medium, Ham's F10 medium (F10), Ham's F-12 medium (F12), Iscove's modified Dulbecco's medium, Mesenchymal Stem Cell Growth Medium (MSCGM), DMEM/F12, RPMI 1640, and Cellgro FREE™. The culture medium may be supplemented with one or more components including, for example, fetal bovine serum (FBS), preferably about 2-15% (v/v); equine serum (ES); human serum(HS); beta-mercaptoethanol (BME or 2-ME), preferably about 0.001% (v/v); one or more growth factors, for example, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), leukocyte inhibitory factor (LIF) and erythropoietin; amino acids, including L-valine; and one or more antibiotic and/or antimycotic agents to control microbial contamination, such as, for example, penicillin G, streptomycin sulfate, amphotericin B, gentamicin, and nystatin, either alone or in combination.

The culture medium preferably comprises Growth Medium (DMEM-low glucose, serum, BME, and an antibiotic agent).

[0060] The cells are seeded in culture vessels at a density to allow cell growth. In a preferred embodiment, the cells are cultured at about 0 to about 5 percent by volume CO₂ in air. In some preferred embodiments, the cells are cultured at about 2 to about 25 percent O₂ in air, preferably about 5 to about 20 percent O₂ in air. The cells preferably are cultured at about 25 to about 40°C and more preferably are cultured at 37°C. The cells are preferably cultured in an incubator.

The medium in the culture vessel can be static or agitated, for example, using a bioreactor. The cells preferably are grown under low oxidative stress (e.g., with addition of glutathione, Vitamin C, Catalase, Vitamin E, N-Acetylcysteine). "Low oxidative stress", as used herein, refers to conditions of no or minimal free radical damage to the cultured cells.

[0061] Methods for the selection of the most appropriate culture medium, medium preparation, and cell culture techniques are well known in the art and are described in a variety of sources, including Doyle et al., (eds.), 1995, CELL & TISSUE CULTURE: LABORATORY PROCEDURES, John Wiley & Sons, Chichester; and Ho and Wang (eds.), 1991, ANIMAL CELL BIOREACTORS, Butterworth-Heinemann, Boston.

[0062] After culturing the isolated cells or tissue fragments for a sufficient period of time, UTCs will have grown out, either as a result of migration from the tissue or cell division, or both. In some embodiments of the invention, UTCs are passaged, or removed to a separate culture vessel containing fresh medium of the same or a different type as that used initially, where the population of cells can be mitotically expanded. The cells of the invention may be used at any point between passage 0 and senescence. The cells preferably are passaged between about 3 and about 25 times, more preferably are passaged about 4 to about 12 times, and preferably are passaged 10 or 11 times. Cloning and/or subcloning may be performed to confirm that a clonal population of cells has been isolated.

[0063] In some aspects of the invention, the different cell types present in umbilical cord tissue are fractionated into subpopulations from which the cells can be isolated. This may be accomplished using standard techniques for cell separation including, but not limited to, enzymatic treatment to dissociate the tissue into its component cells, followed by cloning and selection of specific cell types, for example but not limited to selection based on morphological and/or biochemical markers; selective growth of desired cells (positive selection), selective destruction of unwanted cells (negative selection); separation based upon differential cell agglutinability in the mixed population as, for example, with soybean agglutinin; freeze-thaw procedures; differential adherence properties of the cells in the mixed population; filtration; conventional and zonal centrifugation; centrifugal elutriation (counter-streaming centrifugation); unit gravity separation; countercurrent distribution; electrophoresis; and fluorescence activated cell sorting (FACS). For a review of clonal selection and cell separation techniques, see Freshney, 1994, CULTURE OF ANIMAL CELLS: A MANUAL OF

BASIC TECHNIQUES, 3rd Ed., Wiley-Liss, Inc., New York.

[0064] The culture medium is changed as necessary, for example, by carefully aspirating the medium from the dish, for example, with a pipette, and replenishing with fresh medium. Incubation is continued until a sufficient number or density of cells accumulate in the dish. The original explanted tissue sections may be removed and the remaining cells trypsinized using standard techniques or using a cell scraper. After trypsinization, the cells are collected, removed to fresh medium and incubated as above. In some embodiments, the medium is changed at least once at approximately 24 hours post-trypsinization to remove any floating cells. The cells remaining in culture are considered to be UTCs.

[0065] UTCs may be cryopreserved. Accordingly, in a preferred embodiment described in greater detail below, UTCs for autologous transfer (for either the mother or child) may be derived from appropriate postpartum tissues following the birth of a child, then cryopreserved so as to be available in the event they are later needed for transplantation.

Characteristic of Umbilicus- and Placenta-Derived Cells

[0066] Umbilicus- and placenta-derived cells may be characterized, for example, by growth characteristics (e.g., population doubling capability, doubling time, passages to senescence), karyotype analysis (e.g., normal karyotype; maternal or neonatal lineage), flow cytometry (e.g., FACS analysis), immunohistochemistry and/or immunocytochemistry (e.g., for detection of epitopes), gene expression profiling (e.g., gene chip arrays; polymerase chain reaction (for example, reverse transcriptase PCR, real time PCR, and conventional PCR)), protein arrays, protein secretion (e.g., by plasma clotting assay or analysis of PDC-conditioned medium, for example, by Enzyme Linked ImmunoSorbent Assay (ELISA)), mixed lymphocyte reaction (e.g., as measure of stimulation of PBMCs), and/or other methods known in the art.

[0067] Examples of cells derived from placental tissue were deposited with the American Type Culture Collection (ATCC, Manassas, VA) and assigned ATCC Accession Numbers as follows: (1) strain designation PLA 071003 (P8) was deposited June 15, 2004 and assigned Accession No. PTA-6074; (2) strain designation PLA 071003 (P11) was deposited June 15, 2004 and assigned Accession No. PTA-6075; and (3) strain designation PLA 071003 (P16) was deposited June 16, 2004 and assigned Accession No. PTA-6079. Examples of cells derived from umbilicus tissue were deposited with the American Type Culture Collection on June 10, 2004, and assigned ATCC Accession Numbers as follows: (1) strain designation UMB 022803 (P7) was assigned Accession No. PTA-6067; and (2) strain designation UMB 022803 (P17) was assigned Accession No. PTA-6068.

[0068] In various embodiments, the cells possess one or more of the following growth features (1) they require L-valine for growth in culture; (2) they are capable of growth in atmospheres containing oxygen from about 5% to at least about 20% (3) they have the potential for at least about 40 doublings in culture before reaching senescence; and (4) they attach and expand on a coated or uncoated tissue culture vessel, wherein the coated tissue culture vessel comprises a coating of gelatin, laminin, collagen, polyornithine, vitronectin or fibronectin.

[0069] In certain embodiments the cells possess a normal karyotype, which is maintained as the cells are passaged. Karyotyping is particularly useful for identifying and distinguishing neonatal from maternal cells derived from placenta. Methods for karyotyping are available and known to those of skill in the art.

[0070] In other embodiments, the cells may be characterized by production of certain proteins, including (1) production of at least one of tissue factor, vimentin, and alpha-smooth muscle actin; and (2) production of at least one of CD10, CD 13, CD44, CD73, CD90, PDGFr-alpha, PD-L2 and HLA-A,B,C cell surface markers, as detected by flow cytometry.

[0071] In other embodiments, the UTCs may be characterized by lack of production of at least one of CD31, CD34, CD45, CD80, CD86, CD117, CD141, CD178, B7-H2, HLA-G, and HLA-DR,DP,DQ cell surface markers, as detected by flow cytometry. Particularly preferred are cells that produce at least two of tissue factor, vimentin, and alpha-smooth muscle actin. More preferred are those cells producing all three of the proteins tissue factor, vimentin, and alpha-smooth muscle actin.

[0072] In other embodiments, the cells may be characterized by gene expression, which relative to a human cell that is a fibroblast, a mesenchymal stem cell, or an iliac crest bone marrow cell, is increased for a gene encoding at least one of interleukin 8; reticulon 1; chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha); chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2); chemokine (C-X-C motif) ligand 3; tumor necrosis factor and alpha-induced protein 3.

[0073] In yet other embodiments, the cells may be characterized by gene expression, which relative to a human cell that is a fibroblast, a mesenchymal stem cell, or an iliac crest bone marrow cell, is reduced for a gene encoding at least one of: short stature homeobox 2; heat shock 27 kDa protein 2; chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1); elastin (supravalvular aortic stenosis, Williams-Beuren syndrome); *Homo sapiens* mRNA; cDNA DKFZp586M2022 (from clone DKFZp586M2022); mesenchyme homeo box 2 (growth arrest-specific homeo box); sine oculis homeobox homolog 1 (*Drosophila*); crystallin, alpha B; disheveled associated activator of morphogenesis 2; DKFZP586B2420 protein; similar to neuralin 1; tetranectin (plasminogen binding protein); src homology three (SH3) and cysteine rich domain; cholesterol 25-hydroxylase; runt-related transcription factor 3; interleukin 11 receptor, alpha; pro-collagen C-endopeptidase enhancer; frizzled homolog 7 (*Drosophila*); hypothetical gene BC008967; collagen, type VIII,

alpha 1; tenascin C (hexabrachion); iroquois homeobox protein 5; hephaestin; integrin, beta 8; synaptic vesicle glycoprotein 2; neuroblastoma, suppression of tumorigenicity 1; insulin-like growth factor binding protein 2, 36kDa; *Homo sapiens* cDNA FLJ12280 fis, clone MAMMA1001744; cytokine receptor-like factor 1; potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4; integrin, beta 7; transcriptional co-activator with PDZ-binding motif (TAZ); sine oculis homeobox homolog 2 (Drosophila); KIAA1034 protein; vesicle-associated membrane protein 5 (myobrevin); EGF-containing fibulin-like extracellular matrix protein 1; early growth response 3; distal-less homeo box 5; hypothetical protein FLJ20373; aldo-keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II); biglycan; transcriptional co-activator with PDZ-binding motif (TAZ); fibronectin 1; proenkephalin; integrin, beta-like 1 (with EGF-like repeat domains); *Homo sapiens* mRNA full length insert cDNA clone EUROIMAGE 1968422; EphA3; KIAA0367 protein; natriuretic peptide receptor C/guanylate cyclase C (atrionatriuretic peptide receptor C); hypothetical protein FLJ14054; *Homo sapiens* mRNA; cDNA DKFZp564B222 (from clone DKFZp564B222); BCL2/adenovirus E1B 19kDa interacting protein 3-like; AE binding protein 1; and cytochrome c oxidase subunit VIIa polypeptide 1 (muscle).

[0073] In other embodiments, the cells may be characterized by secretion of at least one of MCP-1, IL-6, IL-8, GCP-2, HGF, KGF, FGF, HB-EGF, BDNF, TPO, MIP1a, RANTES, and TIMP1. In alternative embodiments, the cells may be characterized by lack of secretion of at least one of TGF-beta2, ANG2, PDGFbb, MIP1b, I309, MDC, and VEGF, as detected by ELISA.

[0074] In preferred embodiments, the cell comprises two or more of the above-listed growth, protein/surface marker production, gene expression or substance-secretion characteristics. More preferred are those cells comprising, three, four, or five or more of the characteristics. Still more preferred are cells comprising six, seven, or eight or more of the characteristics. Still more preferred presently are those cells comprising all of above characteristics.

[0075] In one embodiment of the invention, the cells are umbilical cord tissue-derived cells which are isolated from human umbilical cord tissue substantially free of blood, are capable of self-renewal and expansion into culture, have the potential to differentiate into cells of other phenotypes, can undergo at least 40 doublings, and have the following characteristics: (a) express each of CD10, CD13, CD44, CD73, CD90, PDGFr-alpha, PD-L2 and HLA-A,B,C; (b) do not express any of CD31, CD34, CD45, CD80, CD86, CD 117, CD141, CD178, B7-H2, HLA-G, or HLA-DR,DP,DQ; and (c) increased expression of interleukin-8; reticulon 1; and chemokine receptor ligand (C-X-C motif) ligand 3, relative to that of a human cell which is a fibroblast, a mesenchymal stem cell, or an iliac crest bone marrow cell. In one embodiment, these umbilical cord derived cells also have one of more of the following characteristics: (a) secretion of each of the factor MCP-1, MIP1beta, IL-6, IL-8, GCP-2, HGF, KGF, FGF, HB-EGF, BDNF, TPO, RANTES, and TIMP1; and (b) no secretion of any of the factors SDF-1alpha TGF-beta2, ANG2, PDGFbb, MIP1a and VEGF. In another embodiment, these umbilical cord tissue-derived cells do not express hTERT or telomerase.

[0076] Among cells that are presently preferred for use with the invention in several of its aspects are umbilical cord tissue-derived cells having the characteristics described above and more particularly those wherein the cells have normal karyotypes and maintain normal karyotypes with passaging, and further wherein the cells express each of the markers CD10, CD13, CD44, CD73, CD90, PDGFr-alpha, and HLA-A,B,C, wherein the cells produce the immunologically-detectable proteins which correspond to the listed markers. Still more preferred are those cells which in addition to the foregoing do not produce proteins corresponding to any of the markers CD31, CD34, CD45, CD117, CD141, or HLA-DR,DP,DQ, as detected by flow cytometry.

[0077] Certain cells having the potential to differentiate along lines leading to various phenotypes are unstable and thus can spontaneously differentiate. Presently preferred for use with the invention are cells that do not spontaneously differentiate, for example along neural lines. Preferred cells, when grown in Growth Medium, are substantially stable with respect to the cell markers produced on their surface, and with respect to the expression pattern of various genes, for example as determined using an Affymetrix GENECHIP®. The cells remain substantially constant, for example in their surface marker characteristics over passaging, through multiple population doublings.

[0078] However, one feature of the cells is that they may be deliberately induced to differentiate into neural lineage phenotypes by subjecting them to differentiation-inducing cell culture conditions. This may be accomplished by one or more methods known in the art. For instance, as exemplified herein, umbilical cord tissue-derived cells may be plated on flasks coated with laminin in Neurobasal-A medium (Invitrogen, Carlsbad, CA) containing B27 (B27 supplement, Invitrogen), L-glutamine and Penicillin/Streptomycin, the combination of which is referred to herein as Neural Progenitor Expansion (NPE) medium. NPE media may be further supplemented with bFGF and/or EGF. Alternatively, the UTCs may be induced to differentiate *in vitro* by (1) co-culturing the UTCs with neural progenitor cells, or (2) growing the UTCs in neural progenitor cell-conditioned medium.

[0079] Differentiation of the UTCs may be demonstrated by a bipolar cell morphology with extended processes. The induced cell populations may stain positive for the presence of nestin. Differentiated UTCs may be assessed by detection of nestin, TuJ1 (BIII tubulin), GFAP, tyrosine hydroxylase, GABA, O4 and/or MBP. In some embodiments, UTCs have exhibited the ability to form three-dimensional bodies characteristic of neuronal stem cell formation of neurospheres.

UTC populations, modifications, components and products

[0080] Another aspect of the invention features populations of the UTCs described above. In some embodiments, the cell population is heterogeneous. A heterogeneous cell population of the invention may comprise at least about 5%, 5 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 95% UTCs of the invention. The heterogeneous cell populations of the invention may further comprise stem cells or other progenitor cells, such as neural progenitor cells, or it may further comprise fully differentiated neural cells. In some embodiments, the population is substantially homogeneous, i.e., comprises substantially only UTCs (preferably at least about 96%, 97%, 98%, 99% or more UTCs). The homogeneous cell population of the invention comprises umbilicus-derived cells. Homogeneous populations of umbilicus-derived cells 10 are preferably free of cells of maternal lineage. Homogeneity of a cell population may be achieved by any method known in the art, for example, by cell sorting (e.g., flow cytometry) or by clonal expansion in accordance with known methods. Thus, preferred homogeneous UTC populations may comprise a clonal cell line of umbilical cord tissue-derived cells. Such populations are particularly useful when a cell clone with highly desirable functionality has been isolated.

[0081] Also provided herein are populations of cells incubated in the presence of one or more factors, or under 15 conditions, that stimulate stem cell differentiation along a neurogenic pathway. Such factors are known in the art and the skilled artisan will appreciate that determination of suitable conditions for differentiation can be accomplished with routine experimentation. Optimization of such conditions can be accomplished by statistical experimental design and analysis, for example response surface methodology allows simultaneous optimization of multiple variables, for example in a biological culture. Presently preferred factors include, but are not limited to factors, such as growth or trophic factors, 20 demethylating agents, co-culture with neural lineage cells or culture in neural lineage cell-conditioned medium, as well other conditions known in the art to stimulate stem cell differentiation along a neurogenic pathway or lineage (see, e.g., Lang, K.J.D. et al., 2004, J. Neurosci. Res. 76: 184-192; Johe, K.K. et al., 1996, Genes Devel. 10: 3129-3140; Gottlieb, D., 2002, Ann. Rev. Neurosci. 25: 381-407).

[0082] UTCs may also be genetically modified to produce neurotherapeutically useful gene products, or to produce 25 antineoplastic agents for treatment of tumors, for example. Genetic modification may be accomplished using any of a variety of vectors including, but not limited to, integrating viral vectors, e.g., retrovirus vector or adeno-associated viral vectors; non-integrating replicating vectors, e.g., papilloma virus vectors, SV40 vectors, adenoviral vectors; or replication-defective viral vectors. Other methods of introducing DNA into cells include the use of liposomes, electroporation, a particle gun, or by direct DNA injection.

[0083] Hosts cells are preferably transformed or transfected with DNA controlled by or in operative association with, 30 one or more appropriate expression control elements such as promoter or enhancer sequences, transcription terminators, polyadenylation sites, among others, and a selectable marker. Any promoter may be used to drive the expression of the inserted gene. For example, viral promoters include, but are not limited to, the CMV promoter/enhancer, SV 40, papillomavirus, Epstein-Barr virus or elastin gene promoter. In some embodiments, the control elements used to control expression of the gene of interest can allow for the regulated expression of the gene so that the product is synthesized 35 only when needed *in vivo*. If transient expression is desired, constitutive promoters are preferably used in a non-integrating and/or replication-defective vector. Alternatively, inducible promoters could be used to drive the expression of the inserted gene when necessary. Inducible promoters include, but are not limited to, those associated with metallothionein and heat shock proteins.

[0084] Following the introduction of the foreign DNA, engineered cells may be allowed to grow in enriched media and 40 then switched to selective media. The selectable marker in the foreign DNA confers resistance to the selection and allows cells to stably integrate the foreign DNA as, for example, on a plasmid, into their chromosomes and grow to form foci which, in turn, can be cloned and expanded into cell lines. This method can be advantageously used to engineer cell lines that express the gene product.

[0085] The cells of the invention may be genetically engineered to "knock out" or "knock down" expression of factors 45 that promote inflammation or rejection at the implant site. Negative modulatory techniques for the reduction of target gene expression levels or target gene product activity levels are discussed below. "Negative modulation," as used herein, refers to a reduction in the level and/or activity of target gene product relative to the level and/or activity of the target gene product in the absence of the modulatory treatment. The expression of a gene native to a neuron or glial cell can be reduced or knocked out using a number of techniques including, for example, inhibition of expression by inactivating 50 the gene using the homologous recombination technique. Typically, an exon encoding an important region of the protein (or an exon 5' to that region) is interrupted by a positive selectable marker, e.g., neo, preventing the production of normal mRNA from the target gene and resulting in inactivation of the gene. A gene may also be inactivated by creating a deletion in part of a gene, or by deleting the entire gene. By using a construct with two regions of homology to the target gene that are far apart in the genome, the sequences intervening the two regions can be deleted (Mombaerts et al., 1991, Proc. Nat. Acad. Sci. U.S.A. 88:3084-3087). Antisense, DNAzymes, ribozymes, small interfering RNA (siRNA) 55 and other such molecules that inhibit expression of the target gene can also be used to reduce the level of target gene activity. For example, antisense RNA molecules that inhibit the expression of major histocompatibility gene complexes

(HLA) have been shown to be most versatile with respect to immune responses. Still further, triple helix molecules can be utilized in reducing the level of target gene activity. These techniques are described in detail by L.G. Davis et al. (eds), 1994, *BASIC METHODS IN MOLECULAR BIOLOGY*, 2nd ed., Appleton & Lange, Norwalk, CN.

[0086] In other aspects, the disclosure provides cell lysates and cell soluble fractions prepared from UTCs, or heterogeneous or homogeneous cell populations comprising UTCs, as well as UTCs or populations thereof that have been genetically modified or that have been stimulated to differentiate along a neurogenic pathway. Such lysates and fractions thereof have many utilities. Use of the UTC lysate soluble fraction (*i.e.*, substantially free of membranes) *in vivo*, for example, allows the beneficial intracellular milieu to be used allogeneically in a patient without introducing an appreciable amount of the cell surface proteins most likely to trigger rejection, or other adverse immunological responses. Methods of lysing cells are well-known in the art and include various means of mechanical disruption, enzymatic disruption, or chemical disruption, or combinations thereof. Such cell lysates may be prepared from cells directly in their Growth Medium and thus containing secreted growth factors and the like, or may be prepared from cells washed free of medium in, for example, PBS or other solution. Washed cells may be resuspended at concentrations greater than the original population density if preferred.

[0087] In one embodiment, whole cell lysates are prepared, *e.g.*, by disrupting cells without subsequent separation of cell fractions. In another embodiment, a cell membrane fraction is separated from a soluble fraction of the cells by routine methods known in the art, *e.g.*, centrifugation, filtration, or similar methods.

[0088] Cell lysates or cell soluble fractions prepared from populations of umbilical cord tissue-derived cells may be used as is, further concentrated, by for example, ultrafiltration or lyophilization, or even dried, partially purified, combined with pharmaceutically-acceptable carriers or diluents as are known in the art, or combined with other compounds such as biologicals, for example pharmaceutically useful protein compositions. Cell lysates or fractions thereof may be used *in vitro* or *in vivo*, alone or for example, with autologous or syngeneic live cells. The lysates, if introduced *in vivo*, may be introduced locally at a site of treatment, or remotely to provide, for example needed cellular growth factors to a patient.

[0089] In a further embodiment, UTCs can be cultured *in vitro* to produce biological products in high yield. For example, such cells, which either naturally produce a particular biological product of interest (*e.g.*, a trophic factor), or have been genetically engineered to produce a biological product, can be clonally expanded using the culture techniques described herein. Alternatively, cells may be expanded in a medium that induces differentiation to a neural lineage. In either case, biological products produced by the cell and secreted into the medium can be readily isolated from the conditioned medium using standard separation techniques, *e.g.*, such as differential protein precipitation, ion-exchange chromatography, gel filtration chromatography, electrophoresis, and HPLC, to name a few. A "bioreactor" may be used to take advantage of the flow method for feeding, for example, a three-dimensional culture *in vitro*. Essentially, as fresh media is passed through the three-dimensional culture, the biological product is washed out of the culture and may then be isolated from the outflow, as above.

[0090] Alternatively, a biological product of interest may remain within the cell and, thus, its collection may require that the cells be lysed, as described above. The biological product may then be purified using any one or more of the above-listed techniques.

[0091] In other embodiments, the disclosure provides conditioned medium from cultured UTCs for use *in vitro* and *in vivo* as described below. Use of the UTC conditioned medium allows the beneficial trophic factors secreted by the UTCs to be used allogeneically in a patient without introducing intact cells that could trigger rejection, or other adverse immunological responses. Conditioned medium is prepared by culturing cells in a culture medium, then removing the cells from the medium.

[0092] Conditioned medium prepared from populations of umbilical cord tissue-derived cells may be used as is, further concentrated, by for example, ultrafiltration or lyophilization, or even dried, partially purified, combined with pharmaceutically-acceptable carriers or diluents as are known in the art, or combined with other compounds such as biologicals, for example pharmaceutically useful protein compositions. Conditioned medium may be used *in vitro* or *in vivo*, alone or for example, with autologous or syngeneic live cells. The conditioned medium, if introduced *in vivo*, may be introduced locally at a site of treatment, or remotely to provide, for example needed cellular growth or trophic factors to a patient.

[0093] In another embodiment, an extracellular matrix (ECM) produced by culturing UTCs on liquid, solid or semi-solid substrates is prepared, collected and utilized as an alternative to implanting live cells into a subject in need of tissue repair or replacement. UTCs are cultured *in vitro*, on a three dimensional framework as described elsewhere herein, under conditions such that a desired amount of ECM is secreted onto the framework. The comprising the new tissue are removed, and the ECM processed for further use, for example, as an injectable preparation. To accomplish this, cells on the framework are killed and any cellular debris removed from the framework. This process may be carried out in a number of different ways. For example, the living tissue can be flash-frozen in liquid nitrogen without a cryopreservative, or the tissue can be immersed in sterile distilled water so that the cells burst in response to osmotic pressure.

[0094] Once the cells have been killed, the cellular membranes may be disrupted and cellular debris removed by treatment with a mild detergent rinse, such as EDTA, CHAPS or a zwitterionic detergent. Alternatively, the tissue can be enzymatically digested and/or extracted with reagents that break down cellular membranes and allow removal of cell

contents. Example of such enzymes include, but are not limited to, hyaluronidase, dispase, proteases, and nucleases. Examples of detergents include non-ionic detergents such as, for example, alkylaryl polyether alcohol (TRITON X-100), octylphenoxy polyethoxy-ethanol (Rohm and Haas Philadelphia, PA), BRIJ-35, a polyethoxyethanol lauryl ether (Atlas Chemical Co., San Diego, CA), polysorbate 20 (TWEEN 20), a polyethoxyethanol sorbitan monolaureate (Rohm and Haas), polyethylene lauryl ether (Rohm and Haas); and ionic detergents such as, for example, sodium dodecyl sulphate, sulfated higher aliphatic alcohols, sulfonated alkanes and sulfonated alkylarenes containing 7 to 22 carbon atoms in a branched or unbranched chain.

[0095] The collection of the ECM can be accomplished in a variety of ways, depending, for example, on whether the new tissue has been formed on a three-dimensional framework that is biodegradable or non-biodegradable. For example, if the framework is non-biodegradable, the ECM can be removed by subjecting the framework to sonication, high pressure water jets, mechanical scraping, or mild treatment with detergents or enzymes, or any combination of the above.

[0096] If the framework is biodegradable, the ECM can be collected, for example, by allowing the framework to degrade or dissolve in solution. Alternatively, if the biodegradable framework is composed of a material that can itself be injected along with the ECM, the framework and the ECM can be processed *in toto* for subsequent injection. Alternatively, the ECM can be removed from the biodegradable framework by any of the methods described above for collection of ECM from a non-biodegradable framework. All collection processes are preferably designed so as not to denature the ECM.

[0097] After it has been collected, the ECM may be processed further. For example, the ECM can be homogenized to fine particles using techniques well known in the art such as by sonication, so that it can pass through a surgical needle. The components of the ECM can be crosslinked, if desired, by gamma irradiation. Preferably, the ECM can be irradiated between 0.25 to 2 mega rads to sterilize and crosslink the ECM. Chemical crosslinking using agents that are toxic, such as glutaraldehyde, is possible but not generally preferred.

[0098] The amounts and/or ratios of proteins, such as the various types of collagen present in the ECM, may be adjusted by mixing the ECM produced by the cells of the invention with ECM of one or more other cell types. In addition, biologically active substances such as proteins, growth factors and/or drugs, can be incorporated into the ECM. Exemplary biologically active substances include tissue growth factors, such as TGF-beta, and the like, which promote healing and tissue repair at the site of the injection. Such additional agents may be utilized in any of the embodiments described herein above, e.g., with whole cell lysates, soluble cell fractions, or further purified components and products produced by the UTCs.

30 Pharmaceutical compositions comprising UTCs, UTC components or products

[0099] In another aspect, the invention provides pharmaceutical compositions that utilize the UTCs, UTC populations, components and products of UTCs in various methods for treatment of amyotrophic lateral sclerosis. Certain embodiments encompass pharmaceutical compositions comprising live cells (UTCs alone or admixed with other cell types). Other embodiments encompass pharmaceutical compositions comprising UTC cellular components (e.g., cell lysates, soluble cell fractions, conditioned medium, ECM, or components of any of the foregoing) or products (e.g., trophic and other biological factors produced naturally by UTCs or through genetic modification, conditioned medium from UTC culture). In either case, the pharmaceutical composition may further comprise other active agents, such as anti-inflammatory agents, anti-apoptotic agents, antioxidants, growth factors, neurotrophic factors or neuroregenerative or neuroprotective drugs as known in the art.

[0100] Examples of other components that may be added to UTC pharmaceutical compositions include, but are not limited to: (1) other neuroprotective or neurobeneficial drugs; (2) selected extracellular matrix components, such as one or more types of collagen known in the art, and/or growth factors, platelet-rich plasma, and drugs (alternatively, UTCs may be genetically engineered to express and produce growth factors); (3) anti-apoptotic agents (e.g., erythropoietin (EPO), EPO mimetibody, thrombopoietin, insulin-like growth factor (IGF)-I, IGF-II, hepatocyte growth factor, caspase inhibitors); (4) anti-inflammatory compounds (e.g., p38 MAP kinase inhibitors, TGF-beta inhibitors, statins, IL-6 and IL-1 inhibitors, PEMIROLAST, TRANILAST, REMICADE®, SIROLIMUS, and non-steroidal anti-inflammatory drugs (NSAIDS) (such as TEPOXALIN, TOLMETIN, and SUPROFEN); (5) immunosuppressive or immunomodulatory agents, such as calcineurin inhibitors, mTOR inhibitors, antiproliferatives, corticosteroids and various antibodies; (6) antioxidants such as probucol, vitamins C and E, coenzyme Q-10, glutathione, L-cysteine and N-acetylcysteine; and (7) local anesthetics, to name a few.

[0101] Pharmaceutical compositions of the invention comprise UTCs, or components or products thereof, formulated with a pharmaceutically acceptable carrier or medium. Suitable pharmaceutically acceptable carriers include water, salt solution (such as Ringer's solution), alcohols, oils, gelatins, and carbohydrates, such as lactose, amylose, or starch, fatty acid esters, hydroxymethylcellulose, and polyvinyl pyrrolidone. Such preparations can be sterilized, and if desired, mixed with auxiliary agents such as lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, and coloring. Pharmaceutical carriers suitable for use in the present invention are known in the art and are described, for example, in *Pharmaceutical Sciences* (17th Ed., Mack Pub. Co., Easton, PA) and WO

96/05309.

[0102] Typically, but not exclusively, pharmaceutical compositions comprising UTC components or products, but not live cells, are formulated as liquids (or as solid tablets, capsules and the like, when oral delivery is appropriate). These may be formulated for administration by any acceptable route known in the art to achieve delivery of drugs and biological molecules to the target neural tissue, including, but not limited to, oral, nasal, ophthalmic and parenteral, including intravenous. Particular routes of parenteral administration include, but are not limited to, intramuscular, subcutaneous, intraperitoneal, intracerebral, intraventricular, intracerebroventricular, intrathecal, intracisternal, intraspinal and/or peri-spinal routes of administration by delivery via intracranial or intravertebral needles and/or catheters with or without pump devices.

[0103] Pharmaceutical compositions comprising UTC live cells are typically formulated as liquids, semisolids (e.g., gels) or solids (e.g., matrices, scaffolds and the like, as appropriate for neural tissue engineering). Liquid compositions are formulated for administration by any acceptable route known in the art to achieve delivery of live cells to the target neural tissues. Typically, these include injection or infusion into the CNS or PNS, either in a diffuse fashion or targeted to the site of neurological disease or distress, by a route of administration including, but not limited to, intraocular, intracerebral, intraventricular, intracerebroventricular, intrathecal, intracisternal, intraspinal and/or peri-spinal routes of administration by delivery via intracranial or intravertebral needles and/or catheters with or without pump devices.

[0104] Pharmaceutical compositions comprising live cells in a semi-solid or solid carrier are typically formulated for surgical implantation at the site of neurological damage or distress. It will be appreciated that liquid compositions also may be administered by surgical procedures. In particular embodiments, semi-solid or solid pharmaceutical compositions may comprise semi-permeable gels, lattices, cellular scaffolds and the like, which may be non-biodegradable or biodegradable. For example, in certain embodiments, it may be desirable or appropriate to sequester the exogenous cells from their surroundings, yet enable the cells to secrete and deliver biological molecules (e.g. neurotrophic factors) to surrounding neural cells. In these embodiments, cells may be formulated as autonomous implants comprising living UTCs or cell population comprising UTCs surrounded by a non-degradable, selectively permeable barrier that physically separates the transplanted cells from host tissue. Such implants are sometimes referred to as "immunoprotective," as they have the capacity to prevent immune cells and macromolecules from killing the transplanted cells in the absence of pharmacologically induced immunosuppression (for a review of such devices and methods, see, e.g., P.A. Tresco et al., 2000, *Adv. Drug Delivery Rev.* 42: 3-27).

[0105] In one embodiment of the invention, the pharmaceutical compositions comprising umbilical cord tissue-derived cells, the substantially homogenous populations comprising umbilical cord tissue-derived cells or the umbilical cord tissue-derived cells are administered by intravenous and/or intrathecal injection. In another embodiment, the pharmaceutical compositions comprising umbilical cord tissue-derived cells, the substantially homogenous populations comprising umbilical cord tissue-derived cells or the umbilical cord tissue-derived cells are repeatedly administered by intravenous and/or intrathecal injection.

[0106] In other embodiments, different varieties of degradable gels and networks are utilized for the pharmaceutical compositions of the invention. For example, degradable materials particularly suitable for sustained release formulations include biocompatible polymers, such as poly(lactic acid), poly (lactic-co-glycolic acid), methylcellulose, hyaluronic acid, collagen, and the like. The structure, selection and use of degradable polymers in drug delivery vehicles have been reviewed in several publications, including, A. Domb et al., 1992, *Polymers for Advanced Technologies* 3:279.

[0107] In other embodiments, e.g., for repair of large neural lesions, such as a damaged or severed spinal cord or a neural cord of a severed limb, it may be desirable or appropriate to deliver the cells on or in a biodegradable, preferably bioresorbable or bioabsorbable, scaffold or matrix. These typically three-dimensional biomaterials contain the living cells attached to the scaffold, dispersed within the scaffold, or incorporated in an extracellular matrix entrapped in the scaffold. Once implanted into the target region of the body, these implants become integrated with the host tissue, wherein the transplanted cells gradually become established (see, e.g., P.A. Tresco et al., 2000, *supra*; see also D.W. Hutmacher, 2001, *J. Biomater. Sci. Polymer Edn.* 12: 107-174).

[0108] Examples of scaffold or matrix (sometimes referred to collectively as "framework") material that may be used in the present invention include nonwoven mats, porous foams, or self assembling peptides. Nonwoven mats may, for example, be formed using fibers comprised of a synthetic absorbable copolymer of glycolic and lactic acids (PGA/PLA), sold under the trade name VICRYL (Ethicon, Inc., Somerville, NJ). Foams, composed of, for example, poly(epsilon-caprolactone)/poly(glycolic acid) (PCL/PGA) copolymer, formed by processes such as freeze-drying, or lyophilized, as discussed in U.S. Patent No. 6,355,699, also may be utilized. Hydrogels such as self-assembling peptides (e.g., RAD16) may also be used. *In situ*-forming degradable networks are also suitable for use in the invention (see, e.g., Anseth, K.S. et al., 2002, *J. Controlled Release* 78: 199-209; Wang, D. et al., 2003, *Biomaterials* 24: 3969-3980; U.S. Patent Publication 2002/0022676 to He et al.). These materials are formulated as fluids suitable for injection, then may be induced by a variety of means (e.g., change in temperature, pH, exposure to light) to form degradable hydrogel networks *in situ* or *in vivo*.

[0109] In another embodiment, the framework is a felt, which can be composed of a multifilament yarn made from a bioabsorbable material, e.g., PGA, PLA, PCL copolymers or blends, or hyaluronic acid. The yarn is made into a felt

using standard textile processing techniques consisting of crimping, cutting, carding and needling. In another embodiment, cells are seeded onto foam scaffolds that may be composite structures.

[0110] In many of the abovementioned embodiments, the framework may be molded into a useful shape, such as that of the spinal cord with segregated columns for nerve tract repair, for example (Friedman JA et al., 2002, Neurosurgery 51: 742-51). Furthermore, it will be appreciated that UTCs may be cultured on pre-formed, non-degradable surgical or implantable devices, e.g., in a manner corresponding to that used for preparing fibroblast-containing GDC endovascular coils, for instance (Marx, W.F. et al., 2001, Am. J. Neuroradiol. 22: 323-333).

[0111] The matrix, scaffold or device may be treated prior to inoculation of cells in order to enhance cell attachment. For example, prior to inoculation, nylon matrices can be treated with 0.1 molar acetic acid and incubated in polylysine, 10 PBS, and/or collagen to coat the nylon. Polystyrene can be similarly treated using sulfuric acid. The external surfaces of a framework may also be modified to improve the attachment or growth of cells and differentiation of tissue, such as by plasma coating the framework or addition of one or more proteins (e.g., collagens, elastic fibers, reticular fibers), 15 glycoproteins, glycosaminoglycans (e.g., heparin sulfate, chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate, keratin sulfate), a cellular matrix, and/or other materials such as, but not limited to, gelatin, alginates, agar, agarose, and plant gums, among others.

[0112] UTC-containing frameworks are prepared according to methods known in the art. For example, cells can be grown freely in a culture vessel to sub-confluence or confluence, lifted from the culture and inoculated onto the framework. Growth factors may be added to the culture medium prior to, during, or subsequent to inoculation of the cells to trigger 20 differentiation and tissue formation, if desired. Alternatively, the frameworks themselves may be modified so that the growth of cells thereon is enhanced, or so that the risk of rejection of the implant is reduced. Thus, one or more biologically active compounds, including, but not limited to, anti-inflammatories, immunosuppressants or growth factors, may be added to the framework for local release.

Methods of using UTCs, UTC components or products

[0113] UTCs, or cell populations comprising UTCs, or components of or products produced by UTCs, may be used in a variety of ways to support and facilitate repair and regeneration of neural cells and tissues. Such utilities encompass *in vitro*, *ex vivo* and *in vivo* methods. For example, UTCs, or cell populations comprising UTCs, or components of or products produced by UTCs, may be used to treat amyotrophic lateral sclerosis.

In vitro and ex vivo methods:

[0114] In one embodiment, UTCs may be used *in vitro* to screen a wide variety of compounds for effectiveness and cytotoxicity of pharmaceutical agents, growth factors, regulatory factors, and the like. For example, such screening may be performed on substantially homogeneous populations of UTCs to assess the efficacy or toxicity of candidate compounds to be formulated with, or co-administered with, the UTCs, for treatment of a neurodegenerative condition. Alternatively, such screening may be performed on UTCs that have been stimulated to differentiate into a neural cell or neural progenitor cell, for the purpose of evaluating the efficacy of new pharmaceutical drug candidates. In this embodiment, the UTCs are maintained *in vitro* and exposed to the compound to be tested. The activity of a potentially cytotoxic 40 compound can be measured by its ability to damage or kill cells in culture. This may readily be assessed by vital staining techniques. The effect of growth or regulatory factors may be assessed by analyzing the number or robustness of the cultured cells, as compared with cells not exposed to the factors. This may be accomplished using standard cytological and/or histological techniques, including the use of immunocytochemical techniques employing antibodies that define type-specific cellular antigens.

[0115] In a further embodiment, as discussed above, UTCs can be cultured *in vitro* to produce biological products that are either naturally produced by the cells, or produced by the cells when induced to differentiate into neural lineages, or produced by the cells *via* genetic modification. For instance, TIMP1, TPO, KGF, HGF, FGF, HBEGF, BDNF, MIP1b, MCP1, RANTES, I309, TARC, MDC, and IL-8 were found to be secreted from umbilicus-derived cells grown in Growth Medium. TIMP1, TPO, KGF, HGF, HBEGF, BDNF, MIP1a, MCP-1, RANTES, TARC, Eotaxin, and IL-8 were found to be secreted from placenta-derived cells cultured in Growth Medium (see Examples). Some of these trophic factors, such as BDNF and IL-6, have important roles in neural regeneration. Other trophic factors, as yet undetected or unexamined, of use in neural repair and regeneration, are likely to be produced by UTCs and possibly secreted into the medium.

[0116] In this regard, another embodiment features use of UTCs for production of conditioned medium, either from undifferentiated UTCs or from UTCs incubated under conditions that stimulate differentiation into a neural lineage. Such conditioned media are contemplated for use in *in vitro* or *ex vivo* culture of neurogenic precursor cells, or *in vivo* to support transplanted cells comprising homogeneous populations of UTCs or heterogeneous populations comprising UTCs and neural progenitors, for example.

[0117] Yet another embodiment comprises the use of UTC cell lysates, soluble cell fractions or components thereof,

or ECM or components thereof, for a variety of purposes. As mentioned above, some of these components may be used in pharmaceutical compositions. In other embodiments, a cell lysate or ECM is used to coat or otherwise treat substances or devices to be used surgically, or for implantation, or for *ex vivo* purposes, to promote healing or survival of cells or tissues contacted in the course of such treatments.

[0118] As described in Examples 13 and 15, umbilicus and placenta derived-cells have demonstrated the ability to support survival, growth and differentiation of adult neural progenitor cells when grown in co-culture with those cells. Accordingly, in another embodiment, UTCs are used advantageously in co-cultures *in vitro* to provide trophic support to other cells, in particular neural cells and neural progenitors. For co-culture, it may be desirable for the UTCs and the desired other cells to be co-cultured under conditions in which the two cell types are in contact. This can be achieved, for example, by seeding the cells as a heterogeneous population of cells in culture medium or onto a suitable culture substrate. Alternatively, the UTCs can first be grown to confluence, and then will serve as a substrate for the second desired cell type in culture. In this latter embodiment, the cells may further be physically separated, e.g., by a membrane or similar device, such that the other cell type may be removed and used separately, following the co-culture period. Use of UTCs in co-culture to promote expansion and differentiation of neural cell types may find applicability in research and in clinical/therapeutic areas. For instance, UTC co-culture may be utilized to facilitate growth and differentiation of neural cells in culture, for basic research purposes or for use in drug screening assays, for example. UTC co-culture may also be utilized for *ex vivo* expansion of neural progenitors for later administration for therapeutic purposes. For example, neural progenitor cells may be harvested from an individual, expanded *ex vivo* in co-culture with UTCs, then returned to that individual (autologous transfer) or another individual (syngeneic or allogeneic transfer). In these embodiments, it will be appreciated that, following *ex vivo* expansion, the mixed population of cells comprising the UTCs and neural progenitors could be administered to a patient in need of treatment. Alternatively, in situations where autologous transfer is appropriate or desirable, the co-cultured cell populations may be physically separated in culture, enabling removal of the autologous neural progenitors for administration to the patient.

25 **In vivo methods:**

[0119] As set forth in Examples 16 and 17, UTCs have been shown to be effectively transplanted into the body, and to supply lost neural function in an animal model accepted for its predictability of efficacy in humans. These results support a preferred embodiment of the invention, wherein UTCs are used in cell therapy for treating a neurodegenerative condition such as amyotrophic lateral sclerosis. Once transplanted into a target neural location in the body, UTCs may themselves differentiate into one or more neural phenotypes, or they may provide trophic support for neural progenitors and neural cells *in situ*, or they may exert a beneficial effect in both of those fashions, among others.

[0120] UTCs may be administered alone (e.g., as substantially homogeneous populations) or as admixtures with other cells. As described above, UTCs may be administered as formulated in a pharmaceutical preparation with a matrix or scaffold, or with conventional pharmaceutically acceptable carriers. Where UTCs are administered with other cells, they may be administered simultaneously or sequentially with the other cells (either before or after the other cells). Cells that may be administered in conjunction with UTCs include, but are not limited to, neurons, astrocytes, oligodendrocytes, neural progenitor cells, neural stem cells and/or other multipotent or pluripotent stem cells. The cells of different types may be admixed with the UTCs immediately or shortly prior to administration, or they may be co-cultured together for a period of time prior to administration.

[0121] The UTCs may be administered with other neuro-beneficial drugs or biological molecules, or other active agents, such as anti-inflammatory agents, anti-apoptotic agents, antioxidants, growth factors, neurotrophic factors or neuroregenerative or neuroprotective drugs as known in the art. When UTCs are administered with other agents, they may be administered together in a single pharmaceutical composition, or in separate pharmaceutical compositions, simultaneously or sequentially with the other agents (either before or after administration of the other agents).

[0122] Examples of other components that may be administered with UTCs include, but are not limited to: (1) other neuroprotective or neurobeneficial drugs; (2) selected extracellular matrix components, such as one or more types of collagen known in the art, and/or growth factors, platelet-rich plasma, and drugs (alternatively, UTCs may be genetically engineered to express and produce growth factors); (3) anti-apoptotic agents (e.g., erythropoietin (EPO), EPO mimetic-body, thrombopoietin, insulin-like growth factor (IGF)-I, IGF-II, hepatocyte growth factor, caspase inhibitors); (4) anti-inflammatory compounds (e.g., p38 MAP kinase inhibitors, TGF-beta inhibitors, statins, IL-6 and IL-1 inhibitors, PEMIRO-LAST, TRANILAST, REMICADE®, SIROLIMUS, and non-steroidal anti-inflammatory drugs (NSAIDS) (such as TE-POXALIN, TOLMETIN, and SUPROFEN); (5) immunosuppressive or immunomodulatory agents, such as calcineurin inhibitors, mTOR inhibitors, antiproliferatives, corticosteroids and various antibodies; (6) antioxidants such as probucol, vitamins C and E, coenzyme Q-10, glutathione, L-cysteine and N-acetylcysteine; and (7) local anesthetics, to name a few.

[0123] In one embodiment, UTCs are administered as undifferentiated cells, *i.e.*, as cultured in Growth Medium. Alternatively, UTCs may be administered following exposure in culture to conditions that stimulate differentiation toward a desired neural phenotype, e.g., astrocyte, oligodendrocyte or neuron, and more specifically, serotoninergic, dopamin-

ergic, cholinergic, GABA-ergic or glutamatergic neurons (see, e.g., Isacson, O., 2003. *The Lancet (Neurology)* 2: 417-424).

[0124] The cells of the invention may be surgically implanted, injected, delivered (e.g., by way of a catheter or syringe), or otherwise administered directly or indirectly to the site of neurological damage or distress (e.g. for the treatment of amyotrophic lateral sclerosis). Routes of administration of the cells of the invention or compositions thereof include, but are not limited to, intravenous, intramuscular, subcutaneous, intranasal, intracerebral, intraventricular, intracerebroventricular, intrathecal, intracisternal, intraspinal and/or peri-spinal routes of administration by delivery via intracranial or intravertebral needles and/or catheters with or without pump devices.

[0125] When cells are administered in semi-solid or solid devices, surgical implantation into a precise location in the body is typically a suitable means of administration. Liquid or fluid pharmaceutical compositions, however, may be administered to a more general location in the CNS or PNS (e.g., throughout a diffusely affected area, such as would be the case in Parkinson's disease or diffuse ischemic injury, for example), inasmuch as neural progenitor cells have been shown to be capable of extensive migration from a point of entry to the nervous system to a particular location, e.g., by following radial glia or by responding to chemical signals.

[0126] Indeed, this migratory ability of neural stem cells has opened a new avenue for treatment of malignant brain tumors, i.e., use of progenitor cells for delivery of therapeutic genes/gene products for the treatment of these migratory tumors. For example, it has been reported that neural stem cells, when implanted into intracranial gliomas *in vivo* in adult rodents, distribute themselves quickly and extensively through the tumor bed and migrate in juxtaposition to expanding and advancing tumor cells, while continuing to stably express a foreign gene (Aboody, K. et al., 2000, *Proc. Natl. Acad. Sci. USA* 97: 12846-12851). UTCs are also expected to be suitable for this type of use, i.e., UTCs genetically modified to produce an apoptotic or other antineoplastic agent, e.g., IL-12 (Ehtesham, M. et al., 2002, *Cancer Research* 62: 5657-5663) or tumor necrosis factor-related apoptosis-inducing ligand (Ehtesham, M. et al., 2002, *Cancer Research* 62: 7170-7174) may be injected or otherwise administered to a general site of a malignant tumor (e.g., glioblastoma), whereafter the UTCs can migrate to the tumor cells for local delivery of the therapeutic agent.

[0127] Other embodiments encompass methods of treating neurodegenerative conditions (such as e.g. amyotrophic lateral sclerosis) by administering pharmaceutical compositions comprising UTC cellular components (e.g., cell lysates or components thereof) or products (e.g., trophic and other biological factors produced naturally by UTCs or through genetic modification, conditioned medium from UTC culture). Again, these methods may further comprise administering other active agents, such as growth factors, neurotrophic factors or neuroregenerative or neuroprotective drugs as known in the art.

[0128] Dosage forms and regimes for administering UTCs or any of the other pharmaceutical compositions described herein are developed in accordance with good medical practice, taking into account the condition of the individual patient, e.g., nature and extent of the neurodegenerative condition, age, sex, body weight and general medical condition, and other factors known to medical practitioners. Thus, the effective amount of a pharmaceutical composition to be administered to a patient is determined by these considerations as known in the art.

[0129] Because the CNS is a somewhat immunoprivileged tissue, it may not be necessary or desirable to immunosuppress a patient prior to initiation of cell therapy with UTCs. In addition, as set forth in Example 11, UTCs have been shown not to stimulate allogeneic PBMCs in a mixed lymphocyte reaction. Accordingly, transplantation with allogeneic, or even xenogeneic, UTCs may be tolerated in some instances.

[0130] However, in other instances it may be desirable or appropriate to pharmacologically immunosuppress a patient prior to initiating cell therapy. This may be accomplished through the use of systemic or local immunosuppressive agents, or it may be accomplished by delivering the cells in an encapsulated device, as described above. These and other means for reducing or eliminating an immune response to the transplanted cells are known in the art. As an alternative, UTCs may be genetically modified to reduce their immunogenicity, as mentioned above.

[0131] Survival of transplanted UTCs in a living patient can be determined through the use of a variety of scanning techniques, e.g., computerized axial tomography (CAT or CT) scan, magnetic resonance imaging (MRI) or positron emission tomography (PET) scans. Determination of transplant survival can also be done *post mortem* by removing the neural tissue, and examining it visually or through a microscope. Alternatively, cells can be treated with stains that are specific for neural cells or products thereof, e.g., neurotransmitters. Transplanted cells can also be identified by prior incorporation of tracer dyes such as rhodamine- or fluorescein-labeled microspheres, fast blue, ferric microparticles, bisbenzamide or genetically introduced reporter gene products, such as beta-galactosidase or beta-glucuronidase.

[0132] Functional integration of transplanted UTCs into neural tissue of a subject can be assessed by examining restoration of the neural function that was damaged or diseased. Such functions include, but are not limited to motor, cognitive, sensory and endocrine functions, in accordance with procedures well known to neurobiologists and physicians.

Kits and banks comprising UTCs, UTC components or products

[0133] In another aspect, the disclosure provides kits that utilize the UTCs, UTC populations, components and products

of UTCs in various methods for neural regeneration and repair as described above. In one embodiment, the kits are useful for the treatment of amyotrophic lateral sclerosis. Where used for treatment of neurodegenerative conditions, or other scheduled treatment, the kits may include one or more cell populations, including at least UTCs and a pharmaceutically acceptable carrier (liquid, semi-solid or solid). The kits also optionally may include a means of administering the cells, for example by injection. The kits further may include instructions for use of the cells. Kits prepared for field hospital use, such as for military use may include full-procedure supplies including tissue scaffolds, surgical sutures, and the like, where the cells are to be used in conjunction with repair of acute injuries. Kits for assays and *in vitro* methods as described herein may contain one or more of (1) UTCs or components or products of UTCs, (2) reagents for practicing the *in vitro* method, (3) other cells or cell populations, as appropriate, and (4) instructions for conducting the *in vitro* method.

[0134] In yet another aspect, banking of tissues, cells, cellular components and cell populations is disclosed. As discussed above, the cells are readily cryopreserved. The disclosure therefore provides methods of cryopreserving the cells in a bank, wherein the cells are stored frozen and associated with a complete characterization of the cells based on immunological, biochemical and genetic properties of the cells. The frozen cells can be thawed and expanded or used directly for autologous, syngeneic, or allogeneic therapy, depending on the requirements of the procedure and the needs of the patient. Preferably, the information on each cryopreserved sample is stored in a computer, which is searchable based on the requirements of the surgeon, procedure and patient with suitable matches being made based on the characterization of the cells or populations. Preferably, the cells of the invention are grown and expanded to the desired quantity of cells and therapeutic cell compositions are prepared either separately or as co-cultures, in the presence or absence of a matrix or support. While for some applications it may be preferable to use cells freshly prepared, the remainder can be cryopreserved and banked by freezing the cells and entering the information in the computer to associate the computer entry with the samples. Even where it is not necessary to match a source or donor with a recipient of such cells, for immunological purposes, the bank system makes it easy to match, for example, desirable biochemical or genetic properties of the banked cells to the therapeutic needs. Upon matching of the desired properties with a banked sample, the sample is retrieved and prepared for therapeutic use. Cell lysates, ECM or cellular components prepared as described herein may also be cryopreserved or otherwise preserved (e.g., by lyophilization) and banked.

[0135] The following examples are provided to describe the invention in greater detail. They are intended to illustrate, not to limit, the invention.

[0136] As used in the following examples and elsewhere in the specification, the term *Growth Medium* generally refers to a medium sufficient for the culturing of UTCs. In particular, one presently preferred medium for the culturing of the cells of the invention comprises Dulbecco's Modified Essential Media (also abbreviated *DMEM* herein). Particularly preferred is DMEM-low glucose (also *DMEM-LG* herein) (Invitrogen, Carlsbad, CA). The DMEM-low glucose is preferably supplemented with 15% (v/v) fetal bovine serum (e.g. defined fetal bovine serum, Hyclone, Logan UT), antibiotics/antimycotics ((preferably 50-100 Units/milliliter penicillin, 50-100 microgram/milliliter streptomycin, and 0-0.25 microgram/milliliter amphotericin B; Invitrogen, Carlsbad, CA)), and 0.001% (v/v) 2-mercaptoethanol (Sigma, St. Louis MO). As used in the Examples below, *Growth Medium* refers to DMEM-low glucose with 15% fetal bovine serum and antibiotics/antimycotics (when penicillin/streptomycin are included, it is preferably at 50 U/milliliter and 50 microgram/milliliter respectively; when penicillin/streptomycin/amphotericin B are used, it is preferably at 100 U/milliliter, 100 microgram/milliliter and 0.25 microgram/milliliter, respectively). In some cases different growth media are used, or different supplementations are provided, and these are normally indicated in the text as supplementations to *Growth Medium*.

[0137] Also relating to the following examples and used elsewhere in the specification, the term *standard growth conditions* refers to culturing of cells at 37°C, in a standard atmosphere comprising 5% CO₂. While foregoing the conditions are useful for culturing, it is to be understood that such conditions are capable of being varied by the skilled artisan who will appreciate the options available in the art for culturing cells.

[0138] The following abbreviations may appear in the examples and elsewhere in the specification and claims: *ANG2* (or *Ang2*) for angiopoietin 2; *APC* for antigen-presenting cells; *BDNF* for brain-derived neurotrophic factor; *bFGF* for basic fibroblast growth factor; *bid* (*BID*) for "bis in die" (twice per day); *CK18* for cytokeratin 18; *CNS* for central nervous system; *CXC ligand 3* for chemokine receptor ligand 3; *DMEM* for Dulbecco's Minimal Essential Medium; *DMEM:Ig* (or *DMEM:Lg*, *DMEM-LG*) for DMEM with low glucose; *EDTA* for ethylene diamine tetraacetic acid; *EGF* (nor *E*) for epidermal growth factor; *FACS* for fluorescent activated cell sorting; *FBS* for fetal bovine serum; *FGF* (nor *F*) for fibroblast growth factor; *GCP-2* for granulocyte chemotactic protein-2; *GFAP* for glial fibrillary acidic protein; *HB-EGF* for heparin-binding epidermal growth factor; *HCAEC* for Human coronary artery endothelial cells; *HGF* for hepatocyte growth factor; *hMSC* for Human mesenchymal stem cells; *HNF-1alpha* for hepatocyte-specific transcription factor 1 alpha; *HUVEC* for Human umbilical vein endothelial cells; *I309* for a chemokine and the ligand for the CCR8 receptor; *IGF-1* for insulin-like growth factor 1; *IL-6* for interleukin-6; *IL-8* for interleukin 8; *K19* for keratin 19; *K8* for keratin 8; *KGF* for keratinocyte growth factor; *LIF* for leukemia inhibitory factor; *MBP* for myelin basic protein; *MCP-1* for monocyte chemotactic protein 1; *MDC* for macrophage-derived chemokine; *MIP1alpha* for macrophage inflammatory protein 1 alpha; *MIP1beta* for macrophage inflammatory protein 1 beta; *MMP* for matrix metalloprotease (MMP); *MSC* for mesenchymal stem cells; *NHDF* for Normal Human Dermal Fibroblasts; *NPE* for Neural Progenitor Expansion media; *O4* for oligodendrocyte or glial differentiation

marker O4; *PBMC* for Peripheral blood mononuclear cell; *PBS* for phosphate buffered saline; *PDGFbb* for platelet derived growth factor; *PO* for "per os" (by mouth); *PNS* for peripheral nervous system; *Rantes* (or *RANTES*) for regulated on activation, normal T cell expressed and secreted; *rhGDF-5* for recombinant human growth and differentiation factor 5; *SC* for subcutaneously; *SDF-1alpha* for stromal-derived factor 1 alpha; *SHH* for sonic hedgehog; *SOP* for standard operating procedure; *TARC* for thymus and activation-regulated chemokine; *TCP* for Tissue culture plastic; *TCPS* for tissue culture polystyrene; *TGFbeta2* for transforming growth factor beta2; *TGF beta-3* for transforming growth factor beta-3; *TIMP1* for tissue inhibitor of matrix metalloproteinase 1; *TPO* for thrombopoietin; *TuJ1* for BIII Tubulin; *VEGF* for vascular endothelial growth factor; *vWF* for von Willebrand factor; and *alphaFP* for alpha-fetoprotein.

10 EXAMPLE 1

Derivation of Cells from Umbilicus and Placenta Tissue

15 **[0139]** This example describes the preparation cells from placental and umbilical cord tissues. Umbilical cords and placenta were obtained upon birth of either a full term or pre-term pregnancy. Cells were harvested from 5 separate donors of umbilicus and placental tissue. Different methods of cell isolation were tested for their ability to yield cells with: 1) the potential to differentiate into cells with different phenotypes, a characteristic common to stem cells, or 2) the potential to provide trophic factors useful for other cells and tissues.

20 Methods & Materials

25 **[0140] Umbilical cell isolation.** Umbilical cords were obtained from National Disease Research Interchange (NDRI, Philadelphia, PA). The tissues were obtained following normal deliveries. The cell isolation protocol was performed aseptically in a laminar flow hood. To remove blood and debris, the cord was washed in phosphate buffered saline (PBS; Invitrogen, Carlsbad, CA) in the presence of antimycotic and antibiotic (100 units/milliliter penicillin, 100 micrograms/milliliter streptomycin, 0.25 micrograms/milliliter amphotericin B). The tissues were then mechanically dissociated in 150 cm² tissue culture plates in the presence of 50 milliliters of medium (DMEM-Low glucose or DMEM-High glucose; Invitrogen), until the tissue was minced into a fine pulp. The chopped tissues were transferred to 50 milliliter conical tubes (approximately 5 grams of tissue per tube). The tissue was then digested in either DMEM-Low glucose medium or DMEM-High glucose medium, each containing antimycotic and antibiotic as described above. In some experiments, an enzyme mixture of collagenase and dispase was used ("C:D;" collagenase (Sigma, St Louis, MO), 500 Units/milliliter; and dispase (Invitrogen), 50 Units/milliliter in DMEM:-Low glucose medium). In other experiments a mixture of collagenase, dispase and hyaluronidase ("C:D:H") was used (collagenase, 500 Units/milliliter; dispase, 50 Units/milliliter; and hyaluronidase (Sigma), 5 Units/milliliter, in DMEM:-Low glucose). The conical tubes containing the tissue, medium and digestion enzymes were incubated at 37°C in an orbital shaker (Environ, Brooklyn, NY) at 225 rpm for 2 hrs.

30 **[0141]** After digestion, the tissues were centrifuged at 150 x g for 5 minutes, the supernatant was aspirated. The pellet was resuspended in 20 milliliters of Growth Medium (DMEM:Low glucose (Invitrogen), 15 percent (v/v) fetal bovine serum (FBS; defined bovine serum; Lot#AND18475; Hyclone, Logan, UT), 0.001% (v/v) 2-mercaptoethanol (Sigma), 1 milliliter per 100 milliliters of antibiotic/antimycotic as described above. The cell suspension was filtered through a 70-micrometer nylon cell strainer (BD Biosciences). An additional 5 milliliters rinse comprising Growth Medium was passed through the strainer. The cell suspension was then passed through a 40-micrometer nylon cell strainer (BD Biosciences) and chased with a rinse of an additional 5 milliliters of Growth Medium.

35 **[0142]** The filtrate was resuspended in Growth Medium (total volume 50 milliliters) and centrifuged at 150 x g for 5 minutes. The supernatant was aspirated and the cells were resuspended in 50 milliliters of fresh Growth Medium. This process was repeated twice more.

40 **[0143]** Upon the final centrifugation supernatant was aspirated and the cell pellet was resuspended in 5 milliliters of fresh Growth Medium. The number of viable cells was determined using Trypan Blue staining. Cells were then cultured under standard conditions.

45 **[0144]** The cells isolated from umbilical cords were seeded at 5,000 cells/cm² onto gelatin-coated T-75 cm² flasks (Corning Inc., Corning, NY) in Growth Medium with antibiotics/antimycotics as described above. After 2 days (in various experiments, cells were incubated from 2-4 days), spent medium was aspirated from the flasks. Cells were washed with PBS three times to remove debris and blood-derived cells. Cells were then replenished with Growth Medium and allowed to grow to confluence (about 10 days from passage 0) to passage 1. On subsequent passages (from passage 1 to 2 and so on), cells reached sub-confluence (75-85 percent confluence) in 4-5 days. For these subsequent passages, cells were seeded at 5000 cells/cm². Cells were grown in a humidified incubator with 5 percent carbon dioxide and atmospheric oxygen, at 37°C.

50 **[0145] Placental Cell Isolation.** Placental tissue was obtained from NDRI (Philadelphia, PA). The tissues were from a pregnancy and were obtained at the time of a normal surgical delivery. Placental cells were isolated as described for

umbilical cell isolation.

[0146] The following example applies to the isolation of separate populations of maternal-derived and neonatal-derived cells from placental tissue.

[0147] The cell isolation protocol was performed aseptically in a laminar flow hood. The placental tissue was washed in phosphate buffered saline (PBS; Invitrogen, Carlsbad, CA) in the presence of antimycotic and antibiotic (as described above) to remove blood and debris. The placental tissue was then dissected into three sections: top-line (neonatal side or aspect), mid-line (mixed cell isolation neonatal and maternal) and bottom line (maternal side or aspect).

[0148] The separated sections were individually washed several times in PBS with antibiotic/antimycotic to further remove blood and debris. Each section was then mechanically dissociated in 150 cm² tissue culture plates in the presence of 50 milliliters of DMEM/Low glucose, to a fine pulp. The pulp was transferred to 50 milliliter conical tubes. Each tube contained approximately 5 grams of tissue. The tissue was digested in either DMEM-Low glucose or DMEM-High glucose medium containing antimycotic and antibiotic (100 U/milliliter penicillin, 100 micrograms/milliliter streptomycin, 0.25 micrograms/milliliter amphotericin B) and digestion enzymes. In some experiments an enzyme mixture of collagenase and dispase ("C:D") was used containing collagenase (Sigma, St Louis, MO) at 500 Units/milliliter and dispase (Invitrogen) at 50 Units/milliliter in DMEM-Low glucose medium. In other experiments a mixture of collagenase, dispase and hyaluronidase (C:D:H) was used (collagenase, 500 Units/milliliter; dispase, 50 Units/milliliter; and hyaluronidase (Sigma), 5 Units/milliliter in DMEM-Low glucose). The conical tubes containing the tissue, medium, and digestion enzymes were incubated for 2 h at 37°C in an orbital shaker (Environ, Brooklyn, NY) at 225 rpm.

[0149] After digestion, the tissues were centrifuged at 150 x g for 5 minutes, the resultant supernatant was aspirated off. The pellet was resuspended in 20 milliliters of Growth Medium with penicillin/streptomycin/amphotericin B. The cell suspension was filtered through a 70 micrometer nylon cell strainer (BD Biosciences), chased by a rinse with an additional 5 milliliters of Growth Medium. The total cell suspension was passed through a 40 micrometer nylon cell strainer (BD Biosciences) followed with an additional 5 milliliters of Growth Medium as a rinse.

[0150] The filtrate was resuspended in Growth Medium (total volume 50 milliliters) and centrifuged at 150 x g for 5 minutes. The supernatant was aspirated and the cell pellet was resuspended in 50 milliliters of fresh Growth Medium. This process was repeated twice more. After the final centrifugation, supernatant was aspirated and the cell pellet was resuspended in 5 milliliters of fresh Growth Medium. A cell count was determined using the Trypan Blue Exclusion test. Cells were then cultured at standard conditions.

[0151] **LIBERASE™ Cell Isolation.** Cells were isolated from umbilicus tissues in DMEM-Low glucose medium with LIBERASE™ (Boehringer Mannheim Corp., Indianapolis, IN) (2.5 milligrams per milliliter, Blendzyme 3; Roche Applied Sciences, Indianapolis, IN) and hyaluronidase (5 Units/milliliter, Sigma). Digestion of the tissue and isolation of the cells was as described for other protease digestions above, using the LIBERASE™/hyaluronidase mixture in place of the C:D or C:D:H enzyme mixture. Tissue digestion with LIBERASE™ resulted in the isolation of cell populations from umbilicus and placental tissues that expanded readily.

[0152] **Cell isolation using other enzyme combinations.** Procedures were compared for isolating cells from the umbilical cord using differing enzyme combinations. Enzymes compared for digestion included: i) collagenase; ii) dispase; iii) hyaluronidase; iv) collagenase:dispase mixture (C:D); v) collagenase:hyaluronidase mixture (C:H); vi) dispase:hyaluronidase mixture (D:H); and vii) collagenase:dispase:hyaluronidase mixture (C:D:H). Differences in cell isolation utilizing these different enzyme digestion conditions were observed (Table 1-1).

Table 1-1: Isolation of cells from umbilical cord tissue using varying enzyme combinations

Enzyme Digest	Cells Isolated	Cell Expansion
Collagenase	X	X
Dispase	+ (> 10 h)	+
Hyaluronidase	X	X
Collagenase:Dispase	++ (< 3 h)	++
Collagenase:Hyaluronidase	++ (< 3 h)	+
Dispase:Hyaluronidase	+ (> 10 h)	+
Collagenase:Dispase:Hyaluronidase	+++ (< 3 h)	+++

Key: + = good, ++ = very good, +++ = excellent, X = no success under conditions tested

[0153] **Isolation of cells from residual blood in the cords.** Other attempts were made to isolate pools of cells from umbilical cord by different approaches. In one instance umbilical cord was sliced and washed with Growth Medium to

dislodge the blood clots and gelatinous material. The mixture of blood, gelatinous material and Growth Medium was collected and centrifuged at 150 x g. The pellet was resuspended and seeded onto gelatin-coated flasks in Growth Medium. From these experiments a cell population was isolated that readily expanded.

[0154] Isolation of cells from cord blood. Cells have also been isolated from cord blood samples attained from NDRI. The isolation protocol used here was that of International Patent Application PCT/US2002/029971 by Ho et al. (Ho, T. W. et al., WO2003025149 A2). Samples (50 milliliter and 10.5 milliliters, respectively) of umbilical cord blood (NDRI, Philadelphia PA) were mixed with lysis buffer (filter-sterilized 155 mM ammonium chloride, 10 millimolar potassium bicarbonate, 0.1 millimolar EDTA buffered to pH 7.2 (all components from Sigma, St. Louis, MO)). Cells were lysed at a ratio of 1:20 cord blood to lysis buffer. The resulting cell suspension was vortexed for 5 seconds, and incubated for 2 minutes at ambient temperature. The lysate was centrifuged (10 minutes at 200 x g). The cell pellet was resuspended in complete minimal essential medium (Gibco, Carlsbad CA) containing 10 percent fetal bovine serum (Hyclone, Logan UT), 4 millimolar glutamine (Mediatech Herndon, VA), 100 Units penicillin per 100 milliliters and 100 micrograms streptomycin per 100 milliliters (Gibco, Carlsbad, CA). The resuspended cells were centrifuged (10 minutes at 200 x g), the supernatant was aspirated, and the cell pellet was washed in complete medium. Cells were seeded directly into either T75 flasks (Corning, NY), T75 laminin-coated flasks, or T175 fibronectin-coated flasks (both Becton Dickinson, Bedford, MA).

[0155] Isolation of cells using different enzyme combinations and growth conditions. To determine whether cell populations could be isolated under different conditions and expanded under a variety of conditions immediately after isolation, cells were digested in Growth Medium with or without 0.001 percent (v/v) 2-mercaptoethanol (Sigma, St. Louis, MO), using the enzyme combination of C:D:H, according to the procedures provided above. Placental-derived cells so isolated were seeded under a variety of conditions. All cells were grown in the presence of penicillin/streptomycin. (Table 1-2).

Table 1-2: Isolation and culture expansion of cells under varying conditions:

Condition	Medium	15% FBS	BME	Gelatin	20% O ₂	Growth Factors
1	DMEM-Lg	Y	Y	Y	Y	N
2	DMEM-Lg	Y	Y	Y	N (5%)	N
3	DMEM-Lg	Y	Y	N	Y	N
4	DMEM-Lg	Y	Y	N	N (5%)	N
5	DMEM-Lg	N (2%)	Y	N (Laminin)	Y	EGF/FGF (20 ng/mL)
6	DMEM-Lg	N (2%)	Y	N (Laminin)	N (5%)	EGF/FGF (20 ng/mL)
7	DMEM-Lg	N (2%)	Y	N (Fibrone)	Y	PDGF/VEGF
8	DMEM-Lg	N (2%)	Y	N (Fibrone)	N (5%)	PDGF/VEGF
9	DMEM-Lg	Y	N	Y	Y	N
10	DMEM-Lg	Y	N	Y	N (5%)	N
11	DMEM-Lg	Y	N	N	Y	N
12	DMEM-Lg	Y	N	N	N (5%)	N
13	DMEM-Lg	N (2%)	N	N (Laminin)	Y	EGF/FGF (20 ng/mL)
14	DMEM-Lg	N (2%)	N	N (Laminin)	N (5%)	EGF/FGF (20 ng/mL)
15	DMEM-Lg	N (2%)	N	N (Fibrone)	Y	PDGF/VEGF
16	DMEM-Lg	N (2%)	N	N (Fibrone)	N (5%)	PDGF/VEGF

[0156] Isolation of cells using different enzyme combinations and growth conditions. In all conditions cells attached and expanded well between passage 0 and 1 (Table 1-2). Cells in conditions 5-8 and 13-16 were demonstrated to proliferate well up to 4 passages after seeding at which point they were cryopreserved and banked.

55 **Results**

[0157] Cell isolation using different enzyme combinations. The combination of C:D:H, provided the best cell yield following isolation, and generated cells which expanded for many more generations in culture than the other conditions

(Table 1). An expandable cell population was not attained using collagenase or hyaluronidase alone. No attempt was made to determine if this result is specific to the collagen that was tested.

[0158] Isolation of cells using different enzyme combinations and growth conditions. Cells attached and expanded well between passage 0 and 1 under all conditions tested for enzyme digestion and growth (Table 2). Cells in experimental conditions 5-8 and 13-16 proliferated well up to 4 passages after seeding, at which point they were cryopreserved. All cells were banked for further investigation.

[0159] Isolation of cells from residual blood in the cords. Nucleated cells attached and grew rapidly. These cells were analyzed by flow cytometry and were similar to cells obtained by enzyme digestion.

[0160] Isolation of cells from cord blood. The preparations contained red blood cells and platelets. No nucleated cells attached and divided during the first 3 weeks. The medium was changed 3 weeks after seeding and no cells were observed to attach and grow.

[0161] Summary. Populations of cells can be derived from umbilical cord and placental tissue efficiently using the enzyme combination collagenase (a matrix metalloprotease), dispase (a neutral protease) and hyaluronidase (a mucolytic enzyme that breaks down hyaluronic acid). LIBERASE™, which is a Blendzyme, may also be used. Specifically, Blendzyme 3, which is collagenase (4 Wunsch units/g) and thermolysin (1714 casein Units/g) was also used together with hyaluronidase to isolate cells. These cells expanded readily over many passages when cultured in Growth Medium on gelatin coated plastic.

[0162] Cells were also isolated from residual blood in the cords, but not cord blood. The presence of cells in blood clots washed from the tissue, that adhere and grow under the conditions used, may be due to cells being released during the dissection process.

EXAMPLE 2

Growth Characteristics of Umbilicus and Placenta-Derived Cells

[0163] The cell expansion potential of umbilical cord tissue and placental-derived cells was compared to other populations of isolated stem cells. The process of cell expansion to senescence is referred to as Hayflick's limit (Hayflick L. (1974) J Am Geriatr Soc. 22:1-12; Hayflick L. (1974) Geontologist. 14:37-45). Umbilical cord tissue-derived cells are highly suited for therapeutic use because they can be readily expanded to sufficient cell numbers.

Materials and Methods

[0164] Gelatin-coating flasks. Tissue culture plastic flasks were coated by adding 20 milliliters 2% (w/v) porcine gelatin (Type B: 225 Bloom; Sigma, St Louis, MO) to a T75 flask (Corning, Corning, NY) for 20 minutes at room temperature. After removing the gelatin solution, 10 milliliters phosphate-buffered saline (PBS) (Invitrogen, Carlsbad, CA) was added and then aspirated.

[0165] Comparison of expansion potential of UTCs with other cell populations. For comparison of growth expansion potential the following cell populations were utilized; i) Mesenchymal stem cells (MSC; Cambrex, Walkersville, MD); ii) Adipose-derived cells (U.S. Patent No. 6,555,374 B1; U.S. Pub. App. 2004/0058412); iii) Normal dermal skin fibroblasts (cc-2509 lot # 9F0844; Cambrex, Walkersville, MD); iv) Umbilicus-derived cells; and v) Placenta-derived cells (U.S. Pub App. 2004/0048372). Cells were initially seeded at 5,000 cells/cm² on gelatin-coated T75 flasks in Growth Medium with penicillin/streptomycin/amphotericin B. For subsequent passages, cell cultures were treated as follows. After trypsinization, viable cells were counted after Trypan Blue staining. Cell suspension (50 microliters) was combined with Trypan Blue (50 milliliters, Sigma, St. Louis MO). Viable cell numbers were estimated using a hemocytometer.

[0166] Following counting, cells were seeded at 5,000 cells/cm² onto gelatin-coated T 75 flasks in 25 milliliters of fresh Growth Medium. Cells were grown under standard conditions at 37°C. The Growth Medium was changed twice per week. When cells reached about 85 percent confluence they were passaged; this process was repeated until the cells reached senescence.

[0167] At each passage, cells were trypsinized and counted. The viable cell yield, population doubling [$\ln(\text{cell final}/\text{cell initial})/\ln 2$] and doubling time (time in culture (h)/population doubling) were calculated. For the purposes of determining optimal cell expansion, the total cell yield per passage was determined by multiplying the total yield for the previous passage by the expansion factor for each passage (i.e., expansion factor = cell final/cell initial).

[0168] Expansion potential of cell banks at low density. The expansion potential of cells banked at passage 10 was also tested, using a different set of conditions. Normal dermal skin fibroblasts (cc-2509 lot # 9F0844; Cambrex, Walkersville, MD), umbilicus-derived cells, and placenta-derived cells were tested. These cell populations had been banked at passage 10 previously, having been cultured at 5,000 cells/cm² and grown to confluence at each passage to that point. The effect of cell density on the cell populations following cell thaw at passage 10 was determined. Cells were thawed under standard conditions and counted using Trypan Blue staining. Thawed cells were then seeded at 1000

5 cells/cm² in DMEM:Low glucose Growth Medium with antibiotic/antimycotic as described above. Cells were grown under standard atmospheric conditions at 37°C. Growth Medium was changed twice a week and cells were passaged as they reached about 85% confluence. Cells were subsequently passaged until senescence, *i.e.*, until they could not be expanded any further. Cells were trypsinized and counted at each passage. The cell yield, population doubling (\ln (cell final/cell initial)/ \ln 2) and doubling time (time in culture (h)/population doubling). The total cell yield per passage was determined by multiplying total yield for the previous passage by the expansion factor for each passage (*i.e.*, expansion factor = cell final/cell initial).

10 [0169] **Expansion of UTCs at low density from initial cell seeding.** The expansion potential of freshly isolated UTCs under low cell seeding conditions was tested. UTCs were prepared as described herein. Cells were seeded at 1000 cells/cm² and passaged as described above until senescence. Cells were grown under standard atmospheric conditions at 37°C. Growth Medium was changed twice per week. Cells were passaged as they reached about 85% confluence. At each passage, cells were trypsinized and counted by Trypan Blue staining. The cell yield, population doubling (\ln (cell final/cell initial)/ \ln 2) and doubling time (time in culture (h)/population doubling) were calculated for each passage. The total cell yield per passage was determined by multiplying the total yield for the previous passage by the expansion factor for each passage (*i.e.* expansion factor = cell final/cell initial). Cells were grown on gelatin and non-gelatin coated flasks.

15 [0170] **Expansion of clonal neonatal placenta-derived cells.** Cloning was used in order to expand a population of neonatal cells from placental tissue. Following isolation of three differential cell populations from the placenta (as described herein), these cell populations were expanded under standard growth conditions and then karyotyped to reveal the identity of the isolated cell populations. Because the cells were isolated from a mother who delivered a boy, it was straightforward to distinguish between the male and female chromosomes by performing metaphase spreads. These experiments demonstrated that fetal-aspect cells were karyotype positive for neonatal phenotype, mid-layer cells were karyotype positive for both neonatal and maternal phenotypes and maternal-aspect cells were karyotype positive for maternal cells.

20 [0171] **Expansion of cells in low oxygen culture conditions.** It has been demonstrated that low oxygen cell culture conditions can improve cell expansion in certain circumstances (U.S. Pub. App. 2004/0005704). To determine if cell expansion of UTCs could be improved by altering cell culture conditions, cultures of umbilical-derived cells were grown in low oxygen conditions. Cells were seeded at 5000 cells/cm² in Growth Medium on gelatin coated flasks. Cells were initially cultured under standard atmospheric conditions through passage 5, at which point they were transferred to low oxygen (5% O₂) culture conditions.

25 [0172] **Other growth conditions.** In other protocols, cells were expanded on non-coated, collagen-coated, fibronectin-coated, laminin-coated and extracellular matrix protein-coated plates. Cultures have been demonstrated to expand well on these different matrices.

30 **Results**

35 [0173] **Comparison of expansion potential of UTCs with other stem cell and non-stem cell populations.** Both umbilical-derived and placenta-derived cells expanded for greater than 40 passages generating cell yields of > 1E17 cells in 60 days. In contrast, MSCs and fibroblasts senesced after < 25 days and <60 days, respectively. Although adipose-derived cells expanded for almost 60 days, they generated total cell yields of 4.5E12. Thus, when seeded at 5000 cells/cm² under the experimental conditions utilized, postpartum-derived cells expanded much better than the other cell types grown under the same conditions (Table 2-1).

40 **Table 2-1:** Growth characteristics for different cell populations grown to senescence

Cell Type	Senescence	Total Population Doublings	Total Cell Yield
MSC	24 d	8	4.72 E7
Adipose	57 d	24	4.5 E12
Fibroblasts	53 d	26	2.82 E13
Umbilicus	65 d	42	6.15 E17
Placenta	80 d	46	2.49 E19

50 [0174] **Expansion potential of cell banks at low density.** Umbilicus-derived, placenta-derived and fibroblast cells expanded for greater than 10 passages generating cell yields of > 1E11 cells in 60 days (Table 2-2). After 60 days under these conditions the fibroblasts became senescent whereas the umbilicus-derived and placenta-derived cell populations

senesced after 80 days, completing >50 and > 40 population doublings respectively.

Table2- 2: Growth characteristics for different cell populations using low density growth expansion from passage 10 till senescence

Cell Type	Senescence	Total Population Doublings	Total Cell Yield
Fibroblast (P10)	80 d	43.68	2.59 E11
Umbilicus (P10)	80 d	53.6	1.25 E14
Placenta (P10)	60 d	32.96	6.09 E12

[0175] Expansion of UTCs at low density from initial cell seeding. UTCs were expanded at low density (1,000 cells/cm²) on gelatin-coated and uncoated plates or flasks. Growth potential of these cells under these conditions was good. The cells expanded readily in a log phase growth. The rate of cell expansion was similar to that observed when placenta-derived cells were seeded at 5000 cells/cm² on gelatin-coated flasks in Growth Medium. No differences were observed in cell expansion potential between culturing on either uncoated flasks or gelatin-coated flasks. However, cells appeared phenotypically much smaller on gelatin-coated flasks and more larger cell phenotypes were observed on uncoated flasks.

[0176] Expansion of clonal neonatal or maternal placenta-derived cells. A clonal neonatal or maternal cell population can be expanded from placenta-derived cells isolated from the neonatal aspect or the maternal aspect, respectively, of the placenta. Cells are serially diluted and then seeded onto gelatin-coated plates in Growth medium for expansion at 1 cell/well in 96-well gelatin coated plates. From this initial cloning, expansive clones are identified, trypsinized, and reseeded in 12-well gelatin-coated plates in Growth medium and then subsequently passaged into T25 gelatin-coated flasks at 5,000 cells/cm² in Growth medium. Subcloning is performed to ensure that a clonal population of cells has been identified. For subcloning experiments, cells are trypsinized and reseeded at 0.5 cells/well. The sub-clones that grow well are expanded in gelatin-coated T25 flasks at 5,000 cells/cm² /flask. Cells are passaged at 5,000 cells/cm² /T75 flask. The growth characteristics of a clone may be plotted to demonstrate cell expansion. Karyotyping analysis can confirm that the clone is either neonatal or maternal.

[0177] Expansion of cells in low oxygen culture conditions. Cells expanded well under the reduced oxygen conditions, however, culturing under low oxygen conditions did not appear to have a significant effect on cell expansion of UTCs under the conditions used.

[0178] Summary. Cell expansion conditions comprising growing isolated umbilical cord tissue-derived cells at densities of about 5000 cells/cm², in Growth Medium on gelatin-coated or uncoated flasks, under standard atmospheric oxygen, are sufficient to generate large numbers of cells at passage 11. Furthermore, the data suggests that the cells can be readily expanded using lower density culture conditions (e.g. 1000 cells/cm²). umbilical cord tissue-derived cell expansion in low oxygen conditions also facilitates cell expansion, although no incremental improvement in cell expansion potential has yet been observed when utilizing these conditions for growth. Presently, culturing umbilical cord tissue-derived cells under standard atmospheric conditions is preferred for generating large pools of cells. However, when the culture conditions are altered, umbilical cord tissue-derived cell expansion can likewise be altered. This strategy may be used to enhance the proliferative and differentiative capacity of these cell populations.

[0179] Under the conditions utilized, while the expansion potential of MSC and adipose-derived cells is limited, umbilical cord tissue-derived cells expand readily to large numbers.

EXAMPLE 3

Evaluation of Growth Media for Placenta-Derived Cells

[0180] Several cell culture media were evaluated for their ability to support the growth of placenta-derived cells. The growth of placenta-derived cells in normal (20%) and low (5%) oxygen was assessed after 3 days using the MTS colorimetric assay.

Methods & Materials

[0181] Placenta-derived cells at passage 8 (P8) were seeded at 1 x 10³ cells/well in 96 well plates in Growth Medium with penicillin/streptomycin. After 8 hours the medium was changed as described below and cells were incubated in normal (atmospheric) or low (5%, v/v) oxygen at 37°C, 5% CO₂ for 48 hours. MTS was added to the culture medium (Cell Titer 96® AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI) for 3 hours and the absorbance

measured at 490 nanometers (Molecular Devices, Sunnyvale CA).

Table 3-1. Culture Media

Culture Medium	Supplier	Added fetal bovine serum % (v/v)
DMEM low glucose	Gibco Carlsbad CA	0, 2 10
DMEM high glucose	Gibco	0, 2 10
RPMI 1640	Mediatech, Inc. Herndon, VA	0, 2 10
Cell gro-free (Serum-free, Protein-free)	Mediatech, Inc.	-
Ham's F10	Mediatech, Inc.	0, 2 10
MSCGM (complete with serum)	Cambrex, Walkersville, MD	0, 2 10
Complete-serum free w/albumin	Mediatech, Inc.	-
Growth Medium	NA	-
Ham's F12	Mediatech, Inc.	0, 2 10
Iscove's	Mediatech, Inc.	0, 2 10
Basal Medium Eagle's	Mediatech, Inc.	
DMEM/F12 (1:1)	Mediatech, Inc.	0, 2 10

Results

[0182] Standard curves for the MTS assay established a linear correlation between an increase in absorbance and an increase in cell number. The absorbance values obtained were converted into estimated cell numbers and the change (%) relative to the initial seeding was calculated.

[0183] The Effect of Serum. The addition of serum to media at normal oxygen conditions resulted in a reproducible dose-dependent increase in absorbance and thus the viable cell number. The addition of serum to complete MSCGM resulted in a dose-dependent decrease in absorbance. In the media without added serum, cells only grew appreciably in Cellgro FREE™, Ham's F10 and DMEM.

[0184] The Effect of Oxygen. Reduced oxygen appeared to increase the growth rate of cells in Growth Medium, Ham's F10, and MSCGM. In decreasing order of growth, the media resulting in the best growth of the cells were Growth Medium >MSCGM> Iscove's + 10% FBS = DMEM-H + 10% FBS = Ham's F12 + 10% FBS = RPMI 1640 + 10% FBS.

[0185] Summary. Placenta-derived cells may be grown in a variety of culture media in normal or low oxygen. Short term growth of placenta-derived cells was determined in twelve basal media with 0, 2 and 10% (v/v) serum in 5% or atmospheric oxygen. In general, placenta-derived cells did not grow as well in serum-free conditions with the exception of Ham's F10 and Cellgro FREE™, which are also protein-free. Growth in these serum-free media was about 25-33% of the maximal growth observed with media containing 15% serum.

EXAMPLE 4

Growth of Umbilical Cord Tissue and Placenta-Derived Cells in Medium Containing D-Valine

[0186] It has been reported that medium containing D-valine instead of the normal L-valine isoform can be used to selectively inhibit the growth of fibroblast-like cells in culture (Hongpaisan J. 2000. Cell Biol Int. 24:1-7; Sordillo et al. 1988. Cell Biol Int Rep.12:355-64). It was not previously known whether umbilical cord tissue and placenta-derived cells could grow in medium containing D-valine.

Methods & Materials

[0187] Placenta-derived cells (P3), fibroblasts (P9) and umbilical-derived cells (P5) were seeded at 5×10^3 cells/cm² in gelatin-coated T75 flasks (Corning, Corning, NY). After 24 hours the medium was removed and the cells were washed with phosphate buffered saline (PBS) (Gibco, Carlsbad, CA) to remove residual medium. The medium was replaced with a Modified Growth Medium (DMEM with D-valine (special order Gibco), 15% (v/v) dialyzed fetal bovine serum (Hyclone, Logan, UT), 0.001% (v/v) betamercaptoethanol (Sigma), penicillin/streptomycin (Gibco)).

Results

[0188] Placenta-derived, umbilical-derived, and fibroblast cells seeded in the D-valine-containing medium did not proliferate, unlike cells seeded in Growth Medium containing dialyzed serum. Fibroblasts cells changed morphologically, increasing in size and changing shape. All of the cells died and eventually detached from the flask surface after 4 weeks. These results indicate that medium containing D-valine is not suitable for selectively growing postpartum-derived cells.

EXAMPLE 5**Cryopreservation Media for Placenta-Derived Cells**

[0189] Cryopreservation media for the cryopreservation of placenta-derived cells were evaluated.

Methods & Materials

[0190] Placenta-derived cells grown in Growth Medium in a gelatin-coated T75 flask were washed with PBS and trypsinized using 1 milliliter Trypsin/EDTA (Gibco). The trypsinization was stopped by adding 10 milliliters Growth Medium. The cells were centrifuged at 150 x g, supernatant removed, and the cell pellet was resuspended in 1 milliliter Growth Medium. An aliquot of cell suspension, 60 microliters, was removed and added to 60 microliters trypan blue (Sigma). The viable cell number was estimated using a hemocytometer. The cell suspension was divided into four equal aliquots each containing 88×10^4 cells each. The cell suspension was centrifuged and resuspended in 1 milliliter of each media below and transferred into Cryovials (Nalgene).

- 1.) Growth Medium +10% (v/v) DMSO (Hybrimax, Sigma, St. Louis, MO)
- 2.) Cell Freezing medium w/DMSO, w/methyl cellulose, serum-free (C6295, Sigma, St. Louis, MO)
- 3.) Cell Freezing medium serum-free (C2639, Sigma, St. Louis, MO)
- 4.) Cell Freezing Medium w/glycerol (C6039, Sigma, St. Louis, MO) The cells were cooled at approximately -1°C/min overnight in a -80°C freezer using a "Mr Frosty" freezing container according to the manufacturer's instructions (Nalgene, Rochester, NY). Vials of cells were transferred into liquid nitrogen for 2 days before thawing rapidly in a 37°C water bath. The cells were added to 10 milliliters Growth Medium and centrifuged before the cell number and viability was estimated. Cells were seeded onto gelatin-coated flasks at 5,000 cells/cm² to determine whether the cells would attach and proliferate.

Results

[0191] The initial viability of the cells to be cryopreserved was assessed by trypan blue staining to be 100%. The initial viability of the cells to be cryopreserved was assessed by trypan blue staining to be 100%.

[0192] There was a commensurate reduction in cell number with viability for C6295 due to cells lysis. The viable cells cryopreserved in all four solutions attached, divided, and produced a confluent monolayer within 3 days. There was no discernable difference in estimated growth rate.

[0193] **Summary.** The cryopreservation of cells is one procedure available for preparation of a cell bank or a cell product. Four cryopreservation mixtures were compared for their ability to protect human placenta-derived cells from freezing damage. Dulbecco's modified Eagle's medium (DMEM) and 10% (v/v) dimethylsulfoxide (DMSO) is the preferred medium of those compared for cryopreservation of placenta-derived cells.

EXAMPLE 6**Karyotype Analysis of Umbilical Cord Tissue and Placenta-Derived Cells**

[0194] Cell lines used in cell therapy are preferably homogeneous and free from any contaminating cell type. Cells used in cell therapy should have a normal chromosome number (46) and structure. To identify placenta- and umbilicus-derived cell lines that are homogeneous and free from cells of non-postpartum tissue origin, karyotypes of cell samples were analyzed.

Materials and Methods

[0195] UTCs from postpartum tissue of a male neonate were cultured in Growth Medium containing penicillin/streptomycin. Postpartum tissue from a male neonate (X,Y) was selected to allow distinction between neonatal-derived cells

and maternal derived cells (X,X). Cells were seeded at 5,000 cells per square centimeter in Growth Medium in a T25 flask (Corning, Corning, NY) and expanded to 80% confluence. A T25 flask containing cells was filled to the neck with Growth Medium. Samples were delivered to a clinical cytogenetics laboratory by courier (estimated lab to lab transport time is one hour). Cells were analyzed during metaphase when the chromosomes are best visualized. Of twenty cells in metaphase counted, five were analyzed for normal homogeneous karyotype number (two). A cell sample was characterized as homogeneous if two karyotypes were observed. A cell sample was characterized as heterogeneous if more than two karyotypes were observed. Additional metaphase cells were counted and analyzed when a heterogeneous karyotype number (four) was identified.

10 **Results**

[0196] All cell samples sent for chromosome analysis were interpreted as exhibiting a normal appearance. Three of the sixteen cell lines analyzed exhibited a heterogeneous phenotype (XX and XY) indicating the presence of cells derived from both neonatal and maternal origins (Table 6-1). Cells derived from tissue Placenta-N were isolated from the neonatal aspect of placenta. At passage zero, this cell line appeared homogeneous XY. However, at passage nine, the cell line was heterogeneous (XX/XY), indicating a previously undetected presence of cells of maternal origin.

Table 6-1. Results of cell karyotype analysis

Tissue	passage	Metaphase cells counted	Metaphase cells analyzed	number of karyotype	ISCN Karyotype
Placenta	22	20	5	2	46 , XX
Umbilical	23	20	5	2	46, XX
Umbilical	6	20	5	2	46, XY
Placenta	2	20	5	2	46, XX
Umbilical	3	20	5	2	46,XX
Placenta-N	0	20	5	2	46, XY
Placenta-V	0	20	5	2	46, XY
Placenta-M	0	21	5	4	46, XY[18]/46,XX[3]
Placenta-M	4	20	5	2	46,XX
Placenta-N	9	25	5	4	46, XY[5]/46,XX[20]
Placenta-N C1	1	20	5	2	46, XY
Placenta-N C3	1	20	6	4	46, XY[2]/46,XX[18]
Placenta-N C4	1	20	5	2	46, XY
Placenta-N C15	1	20	5	2	46, XY
Placenta-N C20	1	20	5	2	46, XY
Key: N- Neonatal aspect; V- villous region; M- maternal aspect; C- clone					

[0197] **Summary.** Chromosome analysis identified placenta- and umbilicus-derived cells whose karyotypes appeared normal as interpreted by a clinical cytogenetic laboratory. Karyotype analysis also identified cell lines free from maternal cells, as determined by homogeneous karyotype.

EXAMPLE 7

Evaluation of Human Umbilical Cord Tissue and Placenta-Derived Cell Surface Markers by Flow Cytometry

5 [0198] Characterization of cell surface proteins or "markers" by flow cytometry can be used to determine a cell line's identity. The consistency of expression can be determined from multiple donors, and in cells exposed to different processing and culturing conditions. Cell lines isolated from the placenta and umbilicus were characterized (by flow cytometry), providing a profile for the identification of these cell lines.

10 **Materials and Methods**

15 [0199] **Media and culture vessels.** Cells were cultured in Growth Medium (Gibco Carlsbad, CA) with penicillin/streptomycin. Cells were cultured in plasma-treated T75, T150, and T225 tissue culture flasks (Corning, Corning, NY) until confluent. The growth surfaces of the flasks were coated with gelatin by incubating 2% (w/v) gelatin (Sigma, St. Louis, MO) for 20 minutes at room temperature.

20 [0200] **Antibody Staining and flow cytometry analysis.** Adherent cells in flasks were washed in PBS and detached with Trypsin/EDTA. Cells were harvested, centrifuged, and resuspended in 3% (v/v) FBS in PBS at a cell concentration of 1×10^7 per milliliter. In accordance to the manufacturer's specifications, antibody to the cell surface marker of interest (see below) was added to one hundred microliters of cell suspension and the mixture was incubated in the dark for 30 minutes at 4°C. After incubation, cells were washed with PBS and centrifuged to remove unbound antibody. Cells were resuspended in 500 microliter PBS and analyzed by flow cytometry. Flow cytometry analysis was performed with a FACScalibur instrument (Becton Dickinson, San Jose, CA).

25 [0201] The following antibodies to cell surface markers were used.

	Antibody	Manufacture	Catalog Number
30	CD10	BD Pharmingen (San Diego, CA)	555375
	CD13	BD Pharmingen	555394
	CD31	BD Pharmingen	555446
	CD34	BD Pharmingen	555821
	CD44	BD Pharmingen	555478
	CD45RA	BD Pharmingen	555489
	CD73	BD Pharmingen	550257
	CD90	BD Pharmingen	555596
	CD117	BD Pharmingen	340529
	CD141	BD Pharmingen	559781
35	PDGFr-alpha	BD Pharmingen	556002
	HLA-A, B, C	BD Pharmingen	555553
	HLA-DR, DP, DQ	BD Pharmingen	555558
	IgG-FITC	Sigma (St. Louis, MO)	F-6522
40	IgG- PE	Sigma	P-4685

45 [0202] **Placenta and umbilicus comparison.** Placenta-derived cells were compared to umbilicus-derived cells at passage 8.

[0203] **Passage to passage comparison.** Placenta- and umbilicus-derived cells were analyzed at passages 8, 15, and 20.

[0204] **Donor to donor comparison.** To compare differences among donors, placenta-derived cells from different donors were compared to each other, and umbilicus-derived cells from different donors were compared to each other.

50 [0205] **Surface coating comparison.** Placenta-derived cells cultured on gelatin-coated flasks was compared to placenta-derived cells cultured on uncoated flasks. Umbilicus-derived cells cultured on gelatin-coated flasks was compared to umbilicus-derived cells cultured on uncoated flasks.

[0206] **Digestion enzyme comparison.** Four treatments used for isolation and preparation of cells were compared. Cells isolated from placenta by treatment with 1) collagenase; 2) collagenase/disperse; 3) collagenase/hyaluronidase; and 4) collagenase/hyaluronidase/disperse were compared.

55 [0207] **Placental layer comparison.** Cells derived from the maternal aspect of placental tissue were compared to cells derived from the villous region of placental tissue and cells derived from the neonatal fetal aspect of placenta.

Results

[0208] Placenta vs. umbilicus comparison. Placenta- and umbilicus-derived cells analyzed by flow cytometry showed positive expression of CD10, CD13, CD44, CD73, CD 90, PDGFr-alpha and HLA-A, B, C, indicated by the increased values of fluorescence relative to the IgG control. These cells were negative for detectable expression of CD31, CD34, CD45, CD117, CD 141, and HLA-DR, DP, DQ, indicated by fluorescence values comparable to the IgG control. Variations in fluorescence values of positive curves were accounted for. The mean (i.e. CD 13) and range (i.e. CD90) of the positive curves showed some variation, but the curves appeared normal, confirming a homogenous population. Both curves individually exhibited values greater than the IgG control.

[0209] Passage to passage comparison -- placenta-derived cells. Placenta-derived cells at passages 8, 15, and 20 analyzed by flow cytometry all were positive for expression of CD10, CD13, CD44, CD73, CD 90, PDGFr-alpha and HLA-A, B, C, as reflected in the increased value of fluorescence relative to the IgG control. The cells were negative for expression of CD31, CD34, CD45, CD117, CD141, and HLA-DR, DP, DQ having fluorescence values consistent with the IgG control.

[0210] Passage to passage comparison -- umbilicus-derived cells. Umbilicus-derived cells at passage 8, 15, and 20 analyzed by flow cytometry all expressed CD10, CD13, CD44, CD73, CD 90, PDGFr-alpha and HLA-A, B, C, indicated by increased fluorescence relative to the IgG control. These cells were negative for CD31, CD34, CD45, CD117, CD141, and HLA-DR, DP, DQ, indicated by fluorescence values consistent with the IgG control.

[0211] Donor to donor comparison - placenta-derived cells. Placenta-derived cells isolated from separate donors analyzed by flow cytometry each expressed CD10, CD13, CD44, CD73, CD 90, PDGFr-alpha and HLA-A, B, C, with increased values of fluorescence relative to the IgG control. The cells were negative for expression of CD31, CD34, CD45, CD117, CD141, and HLA-DR, DP, DQ as indicated by fluorescence value consistent with the IgG control.

[0212] Donor to donor comparison - umbilicus derived cells. Umbilicus-derived cells isolated from separate donors analyzed by flow cytometry each showed positive expression of CD10, CD13, CD44, CD73, CD 90, PDGFr-alpha and HLA-A, B, C, reflected in the increased values of fluorescence relative to the IgG control. These cells were negative for expression of CD31, CD34, CD45, CD 117, CD141, and HLA-DR, DP, DQ with fluorescence values consistent with the IgG control.

[0213] The effect of surface coating with gelatin on placenta-derived cells. Placenta-derived cells expanded on either gelatin-coated or uncoated flasks analyzed by flow cytometry all expressed of CD10, CD13, CD44, CD73, CD 90, PDGFr-alpha and HLA-A, B, C, reflected in the increased values of fluorescence relative to the IgG control. These cells were negative for expression of CD31, CD34, CD45, CD117, CD141, and HLA-DR, DP, DQ indicated by fluorescence values consistent with the IgG control.

[0214] The effect of surface coating with gelatin on umbilicus-derived cells. Umbilicus-derived cells expanded on gelatin and uncoated flasks analyzed by flow cytometry all were positive for expression of CD10, CD13, CD44, CD73, CD 90, PDGFr-alpha and HLA-A, B, C, with increased values of fluorescence relative to the IgG control. These cells were negative for expression of CD31, CD34, CD45, CD117, CD141, and HLA-DR, DP, DQ, with fluorescence values consistent with the IgG control.

[0215] Effect of enzyme digestion procedure used for preparation of the cells on the cell surface marker profile. Placenta-derived cells isolated using various digestion enzymes analyzed by flow cytometry all expressed CD10, CD13, CD44, CD73, CD 90, PDGFr-alpha and HLA-A, B, C, as indicated by the increased values of fluorescence relative to the IgG control. These cells were negative for expression of CD31, CD34, CD45, CD117, CD141, and HLA-DR, DP, DQ, as indicated by fluorescence values consistent with the IgG control.

[0216] Placental layer comparison. Cells isolated from the maternal, villous, and neonatal layers of the placenta, respectively, analyzed by flow cytometry showed positive expression of CD10, CD13, CD44, CD73, CD 90, PDGFr-alpha and HLA-A, B, C, as indicated by the increased value of fluorescence relative to the IgG control. These cells were negative for expression of CD31, CD34, CD45, CD117, CD141, and HLA-DR, DP, DQ as indicated by fluorescence values consistent with the IgG control.

[0217] Summary. Analysis of placenta- and umbilicus-derived cells by flow cytometry has established of an identity of these cell lines. Placenta- and umbilicus-derived cells are positive for CD10, CD13, CD44, CD73, CD90, PDGFr-alpha, HLA-A,B,C and negative for CD31, CD34, CD45, CD117, CD141 and HLA-DR, DP, DQ. This identity was consistent between variations in variables including the donor, passage, culture vessel surface coating, digestion enzymes, and placental layer. Some variation in individual fluorescence value histogram curve means and ranges was observed, but all positive curves under all conditions tested were normal and expressed fluorescence values greater than the IgG control, thus confirming that the cells comprise a homogenous population that has positive expression of the markers.

EXAMPLE 8**Immunohistochemical Characterization of Umbilical Cord and Placental Tissue Phenotypes**

5 [0218] The phenotypes of cells found within human umbilical cord and placenta were analyzed by immunohistochemistry.

Materials & Methods

10 [0219] **Tissue Preparation.** Human umbilical cord and placenta tissue was harvested and immersion fixed in 4% (w/v) paraformaldehyde overnight at 4°C. Immunohistochemistry was performed using antibodies directed against the following epitopes: vimentin (1:500; Sigma, St. Louis, MO), desmin (1:150, raised against rabbit; Sigma; or 1:300, raised against mouse; Chemicon, Temecula, CA), alpha-smooth muscle actin (SMA; 1:400; Sigma), cytokeratin 18 (CK18; 1:400; Sigma), von Willebrand Factor (vWF; 1:200; Sigma), and CD34 (human CD34 Class III; 1:100; DAKOCytomation, Carpinteria, CA). In addition, the following markers were tested: anti-human GROalpha - PE (1:100; Becton Dickinson, Franklin Lakes, NJ), anti-human GCP-2 (1:100; Santa Cruz Biotech, Santa Cruz, CA), anti-human oxidized LDL receptor 1 (ox-LDL R1; 1:100; Santa Cruz Biotech), and anti-human NOGO-A (1:100; Santa Cruz Biotech). Fixed specimens were trimmed with a scalpel and placed within OCT embedding compound (Tissue-Tek OCT; Sakura, Torrance, CA) on a dry ice bath containing ethanol. Frozen blocks were then sectioned (10 µm thick) using a standard cryostat (Leica Microsystems) and mounted onto glass slides for staining.

15 [0220] **Immunohistochemistry.** Immunohistochemistry was performed similar to previous studies (e.g., Messina, et al., 2003, *Exper. Neurol.* 184: 816-829). Tissue sections were washed with phosphate-buffered saline (PBS) and exposed to a protein blocking solution containing PBS, 4% (v/v) goat serum (Chemicon, Temecula, CA), and 0.3% (v/v) Triton (Triton X-100; Sigma) for 1 hour to access intracellular antigens. In instances where the epitope of interest would be located on the cell surface (CD34, ox-LDL R1), Triton was omitted in all steps of the procedure in order to prevent epitope loss. Furthermore, in instances where the primary antibody was raised against goat (GCP-2, ox-LDL R1, NOGO-A), 3% (v/v) donkey serum was used in place of goat serum throughout the procedure. Primary antibodies, diluted in blocking solution, were then applied to the sections for a period of 4 hours at room temperature. Primary antibody solutions were removed, and cultures washed with PBS prior to application of secondary antibody solutions (1 hour at room temperature) containing block along with goat anti-mouse IgG - Texas Red (1:250; Molecular Probes, Eugene, OR) and/or goat anti-rabbit IgG - Alexa 488 (1:250; Molecular Probes) or donkey anti-goat IgG - FITC (1:150; Santa Cruz Biotech). Cultures were washed, and 10 micromolar DAPI (Molecular Probes) was applied for 10 minutes to visualize cell nuclei.

20 [0221] Following immunostaining, fluorescence was visualized using the appropriate fluorescence filter on an Olympus inverted epi-fluorescent microscope (Olympus, Melville, NY). Positive staining was represented by fluorescence signal above control staining. Representative images were captured using a digital color videocamera and ImagePro software (Media Cybernetics, Carlsbad, CA). For triple-stained samples, each image was taken using only one emission filter at a time. Layered montages were then prepared using Adobe Photoshop software (Adobe, San Jose, CA).

Results

25 [0222] **Umbilical cord characterization.** Vimentin, desmin, SMA, CK18, vWF, and CD34 markers were expressed in a subset of the cells found within umbilical cord. In particular, vWF and CD34 expression were restricted to blood vessels contained within the cord. CD34+ cells were on the innermost layer (lumen side). Vimentin expression was found throughout the matrix and blood vessels of the cord. SMA was limited to the matrix and outer walls of the artery and vein, but not contained with the vessels themselves. CK18 and desmin were observed within the vessels only, desmin being restricted to the middle and outer layers.

30 [0223] **Placenta characterization.** Vimentin, desmin, SMA, CK18, vWF, and CD34 were all observed within the placenta and regionally specific.

35 [0224] **GROalpha, GCP-2, ox-LDL R1, and NOGO-A Tissue Expression.** None of these markers were observed within umbilical cord or placental tissue.

40 [0225] **Summary.** Vimentin, desmin, alpha-smooth muscle actin, cytokeratin 18, von Willebrand Factor, and CD 34 are expressed in cells within human umbilical cord and placenta.

EXAMPLE 9

45 [0226] Affymetrix GENECHIP® arrays were used to compare gene expression profiles of umbilicus- and placenta-

Analysis of Cells using Oligonucleotide Arrays

derived cells with fibroblasts, human mesenchymal stem cells, and another cell line derived from human bone marrow. This analysis provided a characterization of the cells and identified unique molecular markers for these cells.

Materials and Methods

5 [0227] **Isolation and culture of cells.** Human umbilical cords and placenta were obtained from National Disease Research Interchange (NDRI, Philadelphia, PA) from normal full term deliveries with patient consent. The tissues were received and cells were isolated as described in Example 1. Cells were cultured in Growth Medium (using DMEM-LG) on gelatin-coated tissue culture plastic flasks. The cultures were incubated at 37° C with 5 % CO₂.

10 [0228] Human dermal fibroblasts were purchased from Cambrex Incorporated (Walkersville, MD; Lot number 9F0844) and ATCC CRL-1501 (CCD39SK). Both lines were cultured in DMEM/F12 medium (Invitrogen, Carlsbad, CA) with 10% (v/v) fetal bovine serum (Hyclone) and penicillin/streptomycin (Invitrogen). The cells were grown on standard tissue-treated plastic.

15 [0229] Human mesenchymal stem cells (hMSC) were purchased from Cambrex Incorporated (Walkersville, MD; Lot numbers 2F1655, 2F1656 and 2F1657) and cultured according to the manufacturer's specifications in MSCGM Media (Cambrex). The cells were grown on standard tissue cultured plastic at 37 °C with 5 % CO₂.

20 [0230] Human iliac crest bone marrow was received from NDRI with patient consent. The marrow was processed according to the method outlined by Ho, *et al.* (WO03/025149). The marrow was mixed with lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA, pH 7.2) at a ratio of 1 part bone marrow to 20 parts lysis buffer. The cell suspension was vortexed, incubated for 2 minutes at ambient temperature, and centrifuged for 10 minutes at 500 x g. The supernatant was discarded and the cell pellet was resuspended in Minimal Essential Medium-alpha (Invitrogen) supplemented with 10 % (v/v) fetal bovine serum and 4 mM glutamine. The cells were centrifuged again and the cell pellet was resuspended in fresh medium. The viable mononuclear cells were counted using trypan-blue exclusion (Sigma, St. Louis, MO). The mononuclear cells were seeded in tissue-cultured plastic flasks at 5 x 10⁴ cells/cm². The cells were incubated at 37 °C with 5% CO₂ at either standard atmospheric O₂ or at 5% O₂. Cells were cultured for 5 days without a media change. Media and non-adherent cells were removed after 5 days of culture. The adherent cells were maintained in culture.

25 [0231] **Isolation of mRNA and GENECHIP® Analysis.** Actively growing cultures of cells were removed from the flasks with a cell scraper in cold PBS. The cells were centrifuged for 5 minutes at 300 x g. The supernatant was removed and the cells were resuspended in fresh PBS and centrifuged again. The supernatant was removed and the cell pellet was immediately frozen and stored at -80° C. Cellular mRNA was extracted and transcribed into cDNA, which was then transcribed into cRNA and biotin-labeled. The biotin-labeled cRNA was hybridized with HG-U133A GENECHIP® oligonucleotide array (Affymetrix, Santa Clara CA). The hybridization and data collection was performed according to the manufacturer's specifications. Analyses were performed using "Significance Analysis of Microarrays" (SAM) version 1.21 computer software (Stanford University; Tusher, V.G. *et al.*, 2001, Proc. Natl. Acad. Sci. USA 98: 5116-5121).

Results

35 [0232] Fourteen different populations of cells were analyzed. The cells along with passage information, culture substrate, and culture media are listed in Table 9-1.

40 **Table 9-1.** Cells analyzed by the microarray study. Cell lines are listed by identification code along with passage at time of analysis, cell growth substrate and growth medium.

Cell Population	Passage	Substrate	Medium
Umbilicus (022803)	2	Gelatin	DMEM, 15% FBS, 2-ME
Umbilicus (042103)	3	Gelatin	DMEM, 15% FBS, 2-ME
Umbilicus (071003)	4	Gelatin	DMEM, 15% FBS, 2-ME
Placenta (042203)	12	Gelatin	DMEM, 15% FBS, 2-ME
Placenta (042903)	4	Gelatin	DMEM, 15% FBS, 2-ME
Placenta (071003)	3	Gelatin	DMEM, 15% FBS, 2-ME
ICBM (070203) (5% 02)	3	Plastic	MEM, 10% FBS
ICBM (062703) (std. 02)	5	Plastic	MEM, 10% FBS
ICBM (062703) (5% 02)	5	Plastic	MEM, 10% FBS
hMSC (Lot 2F1655)	3	Plastic	MSCGM

(continued)

Cell Population	Passage	Substrate	Medium
hMSC (Lot 2F1656)	3	Plastic	MSCGM
hMSC (Lot 2F 1657)	3	Plastic	MSCGM
hFibroblast (9F0844)	9	Plastic	DMEM-F12, 10% FBS
hFibroblast (CCD39SK)	4	Plastic	DMEM-F12, 10% FBS

[0233] The data were evaluated by a Principle Component Analysis, analyzing the 290 genes that were differentially expressed in the cells. This analysis allows for a relative comparison for the similarities between the populations. Table 9-2 shows the Euclidean distances that were calculated for the comparison of the cell pairs. The Euclidean distances were based on the comparison of the cells based on the 290 genes that were differentially expressed among the cell types. The Euclidean distance is inversely proportional to similarity between the expression of the 290 genes (i.e., the greater the distance, the less similarity exists).

Table 9-2. The Euclidean Distances for the Cell Pairs.

Cell Pair	Euclidean Distance
ICBM-hMSC	24.71
Placenta-umbilical	25.52
ICBM-Fibroblast	36.44
ICBM-placenta	37.09
Fibroblast-MSC	39.63
ICBM-Umbilical	40.15
Fibroblast-Umbilical	41.59
MSC-Placenta	42.84
MSC-Umbilical	46.86
ICBM-placenta	48.41

[0234] Tables 9-3, 9-4, and 9-5 show the expression of genes increased in placenta-derived cells (Table 9-3), increased in umbilicus-derived cells (Table 9-4), and reduced in umbilicus-and placenta-derived cells (Table 9-5). The column entitled "Probe Set ID" refers to the manufacturer's identification code for the sets of several oligonucleotide probes located on a particular site on the chip, which hybridize to the named gene (column "Gene Name"), comprising a sequence that can be found within the NCBI (GenBank) database at the specified accession number (column "NCBI Accession Number").

Table 9-3. Genes shown to have specifically increased expression in the placenta-derived cells as compared to other cell lines assayed

Probe Set ID	Gene Name	NCBI Accession Number
209732_at	C-type (calcium dependent, carbohydrate-recognition domain) lectin, superfamily member 2 (activation-induced)	AF070642
206067_s_at	Wilms tumor 1	NM_024426
207016_s_at	aldehyde dehydrogenase 1 family, member A2	AB015228
206367_at	renin	NM_000537
210004_at	oxidized low density lipoprotein (lectin-like) receptor 1	AF035776
214993_at	<i>Homo sapiens</i> , clone IMAGE:4179671, mRNA, partial cds	AF070642
202178_at	protein kinase C, zeta	NM_002744
209780_at	hypothetical protein DKFZp564F013	AL136883

(continued)

5 **Table 9-3. Genes shown to have specifically increased expression in the placenta-derived cells as compared to other cell lines assayed**

Probe Set ID	Gene Name	NCBI Accession Number
204135_at	downregulated in ovarian cancer 1	NM_014890
213542_at	<i>Homo sapiens</i> mRNA; cDNA DKFZp547K1113 (from clone DKFZp547K1113)	AI246730

15 **Table 9-4. Genes shown to have specifically increased expression in the umbilicus-derived cells as compared to other cell lines assayed**

Probe Set ID	Gene Name	NCBI Accession Number
202859_x_at	interleukin 8	NM_000584
211506_s_at	interleukin 8	AF043337
210222_s_at	reticulon 1	BC000314
204470_at	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity)	NM_001511
206336_at	chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	NM_002993
207850_at	chemokine (C-X-C motif) ligand 3	NM_002090
203485_at	reticulon 1	NM_021136
202644_s_at	tumor necrosis factor, alpha-induced protein 3	NM_006290

35 **Table 9-5. Genes shown to have decreased expression in umbilicus- and placenta-derived cells as compared to other cell lines assayed**

Probe Set ID	Gene name	NCBI Accession Number
210135_s_at	short stature homeobox 2	AF022654.1
205824_at	heat shock 27kDa protein 2	NM_001541.1
209687_at	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	U19495.1
203666_at	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	NM_000609.1
212670_at	elastin (supravalvular aortic stenosis, Williams-Beuren syndrome)	AA479278
213381_at	<i>Homo sapiens</i> mRNA; cDNA DKFZp586M2022 (from clone DKFZp586M2022)	N91149
206201_s_at	mesenchyme homeo box 2 (growth arrest-specific homeo box)	NM_005924.1
205817_at	sine oculis homeobox homolog 1 (<i>Drosophila</i>)	NM_005982.1
209283_at	crystallin, alpha B	AF007162.1
212793_at	dishevelled associated activator of morphogenesis 2	BF513244

(continued)

Table 9-5. Genes shown to have decreased expression in umbilicus- and placenta-derived cells as compared to other cell lines assayed

Probe Set ID	Gene name	NCBI Accession Number
213488_at	DKFZP586B2420 protein	AL050143.1
209763_at	similar to neuralin 1	AL049176
205200_at	ttranectin (plasminogen binding protein)	NM_003278.1
205743_at	src homology three (SH3) and cysteine rich domain	NM_003149.1
200921_s_at	B-cell translocation gene 1, anti-proliferative	NM_001731.1
206932_at	cholesterol 25-hydroxylase	NM_003956.1
204198_s_at	runt-related transcription factor 3	AA541630
219747_at	hypothetical protein FLJ23191	NM_024574.1
204773_at	interleukin 11 receptor, alpha	NM_004512.1
202465_at	procollagen C-endopeptidase enhancer	NM_002593.2
203706_s_at	frizzled homolog 7 (<i>Drosophila</i>)	NM_003507.1
212736_at	hypothetical gene BC008967	BE299456
214587_at	collagen, type VIII, alpha 1	BE877796
201645_at	tenascin C (hexabrachion)	NM_002160.1
210239_at	iroquois homeobox protein 5	U90304.1
203903_s_at	hephaestin	NM_014799.1
205816_at	integrin, beta 8	NM_002214.1
203069_at	synaptic vesicle glycoprotein 2	NM_014849.1
213909_at	<i>Homo sapiens</i> cDNA FLJ12280 fis, clone MAMMA1001744	AU147799
206315_at	cytokine receptor-like factor 1	NM_004750.1
204401_at	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4	NM_002250.1
216331_at	integrin, alpha 7	AK022548.1
209663_s_at	integrin, alpha 7	AF072132.1
213125_at	DKFZP586L151 protein	AW007573
202133_at	transcriptional co-activator with PDZ-binding motif (TAZ)	AA081084
206511_s_at	sine oculis homeobox homolog 2 (<i>Drosophila</i>)	NM_016932.1
213435_at	KIAA1034 protein	AB028957.1
206115_at	early growth response 3	NM_004430.1
213707_s_at	distal-less homeo box 5	NM_005221.3
218181_s_at	hypothetical protein FLJ20373	NM_017792.1
209160_at	aldo-keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II)	AB018580.1
213905_x_at	biglycan	AA845258
201261_x_at	biglycan	BC002416.1

(continued)

Table 9-5. Genes shown to have decreased expression in umbilicus- and placenta-derived cells as compared to other cell lines assayed

Probe Set ID	Gene name	NCBI Accession Number
202132_at	transcriptional co-activator with PDZ-binding motif (TAZ)	AA081084
214701_s_at	fibronectin 1	AJ276395.1
213791_at	proenkephalin	NM_006211.1
205422_s_at	integrin, beta-like 1 (with EGF-like repeat domains)	NM_004791.1
214927_at	<i>Homo sapiens</i> mRNA full length insert cDNA clone EUROIMAGE 1968422	AL359052.1
206070_s_at	EphA3	AF213459.1
212805_at	KIAA0367 protein	AB002365.1
219789_at	natriuretic peptide receptor C/guanylate cyclase C (atrionatriuretic peptide receptor C)	AI628360
219054_at	hypothetical protein FLJ14054	NM_024563.1
213429_at	<i>Homo sapiens</i> mRNA; cDNA DKFZp564B222 (from clone DKFZp564B222)	AW025579
204929_s_at	vesicle-associated membrane protein 5 (myobrevin)	NM_006634.1
201843_s_at	EGF-containing fibulin-like extracellular matrix protein 1	NM_004105.2
221478_at	BCL2/adenovirus E1B 19kDa interacting protein 3-like	AL132665.1
201792_at	AE binding protein 1	N_001129.2
204570_at	cytochrome c oxidase subunit VIIa polypeptide 1 (muscle)	NM_001864.1
201621_at	neuroblastoma, suppression of tumorigenicity 1	NM_005380.1
202718_at	insulin-like growth factor binding protein 2, 36kDa	NM_000597.1

[0235] Tables 9-6, 9-7, and 9-8 show the expression of genes increased in human fibroblasts (Table 9-6), ICBM cells (Table 9-7), and MSCs (Table 9-8).

Table 9-6. Genes that were shown to have increased expression in fibroblasts as compared to the other cell lines assayed.

dual specificity phosphatase 2
KIAA0527 protein
Homo sapiens cDNA: FLJ23224 fis, clone ADSU02206
dynein, cytoplasmic, intermediate polypeptide 1
ankyrin 3, node of Ranvier (ankyrin G)
inhibin, beta A (activin A, activin AB alpha polypeptide)
ectonucleotide pyrophosphatase/phosphodiesterase 4 (putative function)
KIAA1053 protein
microtubule-associated protein 1A
zinc finger protein 41

(continued)

Table 9-6. Genes that were shown to have increased expression in fibroblasts as compared to the other cell lines assayed.

5	HSPC019 protein
	Homo sapiens cDNA: FLJ23564 fis, clone LNG10773
	Homo sapiens mRNA; cDNA DKFZp564A072 (from clone DKFZp564A072)
10	LIM protein (similar to rat protein kinase C-binding enigma)
	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase complex-associated protein
	hypothetical protein FLJ22004
15	Human (clone CTG-A4) mRNA sequence
	ESTs, Moderately similar to cytokine receptor-like factor 2; cytokine receptor CRL2 precursor [Homo sapiens]
	transforming growth factor, beta 2
	hypothetical protein MGC29643
20	antigen identified by monoclonal antibody MRC OX-2
	putative X-linked retinopathy protein

Table 9-7. Genes that were shown to have increased expression in the ICBM-derived cells as compared to the other cell lines assayed

25	•cardiac ankyrin repeat protein
	•MHC class I region ORF
	ointegрин, alpha 10
30	•hypothetical protein FLJ22362
	•UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3 (GalNAc-T3)
	•interferon-induced protein 44
	•SRY (sex determining region Y)-box 9 (campomelic dysplasia, autosomal sex-reversal)
35	•keratin associated protein 1-1
	•hippocalcin-like 1
	•jagged 1 (Alagille syndrome)
	•proteoglycan 1, secretory granule

Table 9-8. Genes that were shown to have increased expression in the MSC cells as compared to the other cell lines assayed.

40	•interleukin 26
	•maltase-glucoamylase (alpha-glucosidase)
	•nuclear receptor subfamily 4, group A, member 2
	•v-fos FBJ murine osteosarcoma viral oncogene homolog
45	•hypothetical protein DC42
	•nuclear receptor subfamily 4, group A, member 2
	•FBJ murine osteosarcoma viral oncogene homolog B
	•WNT1 inducible signaling pathway protein 1
	•MCF.2 cell line derived transforming sequence
50	•potassium channel, subfamily K, member 15
	•cartilage paired-class homeoprotein 1
	•Homo sapiens cDNA FLJ12232 fis, clone MAMMA1001206
55	•Homo sapiens cDNA FLJ34668 fis, clone LIVER2000775

(continued)

Table 9-8. Genes that were shown to have increased expression in the MSC cells as compared to the other cell lines assayed.

5
-jun B proto-oncogene
•B-cell CLL/lymphoma 6 (zinc finger protein 51)
•zinc finger protein 36, C3H type, homolog (mouse)

10 **[0236] Summary.** The present examination was performed to provide a molecular characterization of the cells derived from umbilical cord and placenta. This analysis included cells derived from three different umbilical cords and three different placentas. The examination also included two different lines of dermal fibroblasts, three lines of mesenchymal stem cells, and three lines of iliac crest bone marrow cells. The mRNA that was expressed by these cells was analyzed using an oligonucleotide array that contained probes for 22,000 genes. Results showed that 290 genes are differentially expressed in these five different cell types. These genes include ten genes that are specifically increased in the placenta-derived cells and seven genes specifically increased in the umbilical cord-derived cells. Fifty-four genes were found to have specifically lower expression levels in placenta and umbilical cord, as compared with the other cell types. The expression of selected genes has been confirmed by PCR (see the example that follows). These results demonstrate that the cells have a distinct gene expression profile, for example, as compared to bone marrow-derived cells and fibroblasts.

EXAMPLE 10

Cell Markers in Umbilical Cord Tissue and Placental-Derived Cells

25 **[0237]** In the preceding example, similarities and differences in cells derived from the human placenta and the human umbilical cord were assessed by comparing their gene expression profiles with those of cells derived from other sources (using an oligonucleotide array). Six "signature" genes were identified: oxidized LDL receptor 1, interleukin-8, rennin, reticulon, chemokine receptor ligand 3 (CXC ligand 3), and granulocyte chemotactic protein 2 (GCP-2). These "signature" genes were expressed at relatively high levels in the cells.

30 **[0238]** The procedures described in this example were conducted to verify the microarray data and find concordance/discordance between gene and protein expression, as well as to establish a series of reliable assay for detection of unique identifiers for placenta- and umbilicus-derived cells.

35 **Methods & Materials**

[0239] Cells. Placenta-derived cells (three isolates, including one isolate predominately neonatal as identified by karyotyping analysis), umbilicus-derived cells (four isolates), and Normal Human Dermal Fibroblasts (NHDF; neonatal and adult) grown in Growth Medium with penicillin/streptomycin in a gelatin-coated T75 flask. Mesenchymal Stem Cells (MSCs) were grown in Mesenchymal Stem Cell Growth Medium Bullet kit (MSCGM; Cambrex, Walkerville, MD).

40 **[0240]** For the IL-8 protocol, cells were thawed from liquid nitrogen and plated in gelatin-coated flasks at 5,000 cells/cm², grown for 48 hours in Growth Medium and then grown for further 8 hours in 10 milliliters of serum starvation medium [DMEM -low glucose (Gibco, Carlsbad, CA), penicillin/streptomycin (Gibco, Carlsbad, CA) and 0.1% (w/v) Bovine Serum Albumin (BSA; Sigma, St. Louis, MO)]. After this treatment RNA was extracted and the supernatants were centrifuged at 150 x g for 5 minutes to remove cellular debris. Supernatants were then frozen at -80°C for ELISA analysis.

45 **[0241] Cell culture for ELISA assay.** Cells derived from placenta and umbilicus, as well as human fibroblasts derived from human neonatal foreskin were cultured in Growth Medium in gelatin-coated T75 flasks. Cells were frozen at passage 11 in liquid nitrogen. Cells were thawed and transferred to 15-milliliter centrifuge tubes. After centrifugation at 150 x g for 5 minutes, the supernatant was discarded. Cells were resuspended in 4 milliliters culture medium and counted. Cells were grown in a 75 cm² flask containing 15 milliliters of Growth Medium at 375,000 cell/flask for 24 hours. The medium was changed to a serum starvation medium for 8 hours. Serum starvation medium was collected at the end of incubation, centrifuged at 14,000 x g for 5 minutes (and stored at -20 °C).

50 **[0242]** To estimate the number of cells in each flask, 2 milliliters of trypsin/EDTA (Gibco, Carlsbad, CA) was added each flask. After cells detached from the flask, trypsin activity was neutralized with 8 milliliters of Growth Medium. Cells were transferred to a 15 milliliters centrifuge tube and centrifuged at 150 x g for 5 minutes. Supernatant was removed and 1 milliliter Growth Medium was added to each tube to resuspend the cells. Cell number was estimated using a hemocytometer.

55 **[0243] ELISA assay.** The amount of IL-8 secreted by the cells into serum starvation medium was analyzed using

ELISA assays (R&D Systems, Minneapolis, MN). All assays were tested according to the instructions provided by the manufacturer.

[0244] Total RNA isolation. RNA was extracted from confluent umbilicus and placenta-derived cells and fibroblasts or for IL-8 expression from cells treated as described above. Cells were lysed with 350 microliters buffer RLT containing beta-mercaptoethanol (Sigma, St. Louis, MO) according to the manufacturer's instructions (RNeasy Mini Kit; Qiagen, Valencia, CA). RNA was extracted according to the manufacturer's instructions (RNeasy Mini Kit; Qiagen, Valencia, CA) and subjected to DNase treatment (2.7 U/sample) (Sigma St. Louis, MO). RNA was eluted with 50 microliters DEPC-treated water and stored at -80°C.

[0245] Reverse transcription. RNA was also extracted from human placenta and umbilicus. Tissue (30 milligram) was suspended in 700 microliters of buffer RLT containing 2-mercaptoethanol. Samples were mechanically homogenized and the RNA extraction proceeded according to manufacturer's specification. RNA was extracted with 50 microliters of DEPC-treated water and stored at -80°C. RNA was reversed transcribed using random hexamers with the TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA) at 25°C for 10 minutes, 37°C for 60 minutes, and 95°C for 10 minutes. Samples were stored at -20°C.

[0246] Genes identified by cDNA microarray as uniquely regulated in postpartum cells (signature genes - including oxidized LDL receptor, interleukin-8, rennin and reticulon), were further investigated using real-time and conventional PCR.

[0247] Real-time PCR. PCR was performed on cDNA samples using Assays-on-Demand™ gene expression products: oxidized LDL receptor (Hs00234028); rennin (Hs00166915); reticulon (Hs00382515); CXC ligand 3 (Hs00171061); GCP-2 (Hs00605742); IL-8 (Hs00174103); and GAPDH (Applied Biosystems, Foster City, CA) were mixed with cDNA and TaqMan Universal PCR master mix according to the manufacturer's instructions (Applied Biosystems, Foster City, CA) using a 7000 sequence detection system with ABI Prism 7000 SDS software (Applied Biosystems, Foster City, CA). Thermal cycle conditions were initially 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. PCR data was analyzed according to manufacturer's specifications (User Bulletin #2 from Applied Biosystems for ABI Prism 7700 Sequence Detection System).

[0248] Conventional PCR. Conventional PCR was performed using an ABI PRISM 7700 (Perkin Elmer Applied Biosystems, Boston, Massachusetts, USA) to confirm the results from real-time PCR. PCR was performed using 2 microliters of cDNA solution, 1 × AmpliTaq Gold universal mix PCR reaction buffer (Applied Biosystems, Foster City, CA) and initial denaturation at 94°C for 5 minutes. Amplification was optimized for each primer set. For IL-8, CXC ligand 3, and reticulon (94°C for 15 seconds, 55°C for 15 seconds and 72°C for 30 seconds for 30 cycles); for rennin (94°C for 15 seconds, 53°C for 15 seconds and 72°C for 30 seconds for 38 cycles); for oxidized LDL receptor and GAPDH (94°C for 15 seconds, 55°C for 15 seconds and 72°C for 30 seconds for 33 cycles). Primers used for amplification are listed in Table 1. Primer concentration in the final PCR reaction was 1 micromolar except for GAPDH, which was 0.5 micromolar. GAPDH primers were the same as real-time PCR, except that the manufacturer's TaqMan probe was not added to the final PCR reaction. Samples were run on 2% (w/v) agarose gel and stained with ethidium bromide (Sigma, St. Louis, MO). Images were captured using a 667 Universal Twinpack film (VWR International, South Plainfield, NJ) using a focal-length Polaroid camera (VWR International, South Plainfield, NJ).

Table 10-1: Primers used

40	Primer name	Primers
	Oxidized LDL receptor	S: 5'-GAGAAATCCAAAGAGCAAATGG-3' (SEQ ID NO:1) A: 5'-AGAATGGAAAATCTGGAATAGG-3' (SEQ ID NO:2)
	Renin	S: 5'-TCTTCGATGCTTCGGATTCC-3' (SEQ ID NO:3) A: 5'-GAATTCTCGGAATCTCTGTTG-3' (SEQ ID NO:4)
45	Reticulon	S: 5'- TTACAAGCAGTCAGAAAACC-3' (SEQ ID NO:5) A: 5'- AGTAAACATTGAAACCACAGCC-3' (SEQ ID NO:6)
	Interleukin-8	S: 5'- TCTGCAGCTCTGTGAAAGG-3' (SEQ ID NO:7) A: 5'- CTTCAAAAATCTCTCCACAACC- 3' (SEQ ID NO:8)
50	Chemokine (CXC) ligand 3	S: 5'- CCCACGCCACGCTCTCC-3' (SEQ ID NO:9) A: 5'-TCCTGTCAGTTGGTGCTCC -3' (SEQ ID NO:10)

[0249] Immunofluorescence. Derived cells were fixed with cold 4% (w/v) paraformaldehyde (Sigma-Aldrich, St. Louis, MO) for 10 minutes at room temperature. One isolate each of umbilicus- and placenta-derived cells at passage 0 (P0) (directly after isolation) and passage 11 (P11) (two isolates of placenta-derived, two isolates of umbilicus-derived cells) and fibroblasts (P11) were used. Immunocytochemistry was performed using antibodies directed against the following epitopes: vimentin (1:500, Sigma, St. Louis, MO), desmin (1:150; Sigma - raised against rabbit; or 1:300; Chemicon,

Temecula, CA - raised against mouse,), alpha-smooth muscle actin (SMA; 1:400; Sigma), cytokeratin 18 (CK18; 1:400; Sigma), von Willebrand Factor (vWF; 1:200; Sigma), and CD34 (human CD34 Class III; 1:100; DAKOCytomation, Carpinteria, CA). In addition, the following markers were tested on passage 11 postpartum cells: anti-human GRO alpha - PE (1:100; Becton Dickinson, Franklin Lakes, NJ), anti-human GCP-2 (1:100; Santa Cruz Biotech, Santa Cruz, CA), anti-human oxidized LDL receptor 1 (ox-LDL R1; 1:100; Santa Cruz Biotech), and anti-human NOGA-A (1:100; Santa Cruz, Biotech).

[0250] Cultures were washed with phosphate-buffered saline (PBS) and exposed to a protein blocking solution containing PBS, 4% (v/v) goat serum (Chemicon, Temecula, CA), and 0.3% (v/v) Triton (Triton X-100; Sigma, St. Louis, MO) for 30 minutes to access intracellular antigens. Where the epitope of interest was located on the cell surface (CD34, ox-LDL R1), Triton X-100 was omitted in all steps of the procedure in order to prevent epitope loss. Furthermore, in instances where the primary antibody was raised against goat (GCP-2, ox-LDL R1, NOGO-A), 3% (v/v) donkey serum was used in place of goat serum throughout. Primary antibodies, diluted in blocking solution, were then applied to the cultures for a period of 1 hour at room temperature. The primary antibody solutions were removed and the cultures were washed with PBS prior to application of secondary antibody solutions (1 hour at room temperature) containing block along with goat anti-mouse IgG - Texas Red (1:250; Molecular Probes, Eugene, OR) and/or goat anti-rabbit IgG - Alexa 488 (1:250; Molecular Probes) or donkey anti-goat IgG - FITC (1:150, Santa Cruz Biotech). Cultures were then washed and 10 micromolar DAPI (Molecular Probes) applied for 10 minutes to visualize cell nuclei.

[0251] Following immunostaining, fluorescence was visualized using an appropriate fluorescence filter on an Olympus inverted epi-fluorescent microscope (Olympus, Melville, NY). In all cases, positive staining represented fluorescence signal above control staining where the entire procedure outlined above was followed with the exception of application of a primary antibody solution. Representative images were captured using a digital color videocamera and ImagePro software (Media Cybernetics, Carlsbad, CA). For triple-stained samples, each image was taken using only one emission filter at a time. Layered montages were then prepared using Adobe Photoshop software (Adobe, San Jose, CA).

[0252] **Preparation of cells for FACS analysis.** Adherent cells in flasks were washed in phosphate buffered saline (PBS) (Gibco, Carlsbad, CA) and detached with Trypsin/EDTA (Gibco, Carlsbad, CA). Cells were harvested, centrifuged, and re-suspended 3% (v/v) FBS in PBS at a cell concentration of 1×10^7 per milliliter. One hundred microliter aliquots were delivered to conical tubes. Cells stained for intracellular antigens were permeabilized with Perm/Wash buffer (BD Pharmingen, San Diego, CA). Antibody was added to aliquots as per manufactures specifications and the cells were incubated for in the dark for 30 minutes at 4°C. After incubation, cells were washed with PBS and centrifuged to remove excess antibody. Cells requiring a secondary antibody were resuspended in 100 microliters of 3% FBS. Secondary antibody was added as per manufactures specification and the cells were incubated in the dark for 30 minutes at 4°C. After incubation, cells were washed with PBS and centrifuged to remove excess secondary antibody. Washed cells were resuspended in 0.5 milliliters PBS and analyzed by flow cytometry. The following antibodies were used: oxidized LDL receptor 1 (sc-5813; Santa Cruz, Biotech), GROa (555042; BD Pharmingen, Bedford, MA), Mouse IgG1 kappa, (P-4685 and M-5284; Sigma), Donkey against Goat IgG (sc-3743; Santa Cruz, Biotech.). Flow cytometry analysis was performed with FACScalibur (Becton Dickinson San Jose, CA).

Results

[0253] Results of real-time PCR for selected "signature" genes performed on cDNA from cells derived from human placenta, adult and neonatal fibroblasts and Mesenchymal Stem Cells (MSCs) indicate that both oxidized LDL receptor and rennin were expressed at higher level in the placenta-derived cells as compared to other cells. The data obtained from real-time PCR were analyzed by the $\Delta\Delta CT$ method and expressed on a logarithmic scale. Levels of reticulon and oxidized LDL receptor expression were higher in umbilicus-derived cells as compared to other cells. No significant difference in the expression levels of CXC ligand 3 and GCP-2 were found between placenta-derived cells and controls. The results of real-time PCR were confirmed by conventional PCR. Sequencing of PCR products further validated these observations. No significant difference in the expression level of CXC ligand 3 was found between placenta-derived cells and controls using conventional PCR CXC ligand 3 primers listed above.

[0254] The production of the cytokine, IL-8 in postpartum was elevated in both Growth Medium-cultured and serum-starved derived cells. All real-time PCR data was validated with conventional PCR and by sequencing PCR products.

[0255] When supernatants of cells grown in serum-free medium were examined for the presence of IL-8, the highest amounts were detected in media derived from umbilical cells and some isolates of placenta cells (Table 10-1). No IL-8 was detected in medium derived from human dermal fibroblasts.

55 **Table 10-1: IL-8 protein amount measured by ELISA**

Cell type	IL-8
hFibro	ND

(continued)

Cell type	IL-8
Placenta Isolate 1	ND
Umb Isolate 1	2058.42 ± 144.67
Placenta Isolate 2	ND
Umb Isolate 2	2368.86 ± 22.73
Placenta Isolate3 (normal O ₂)	17.27 ± 8.63
Placenta Isolate 3 (lowO ₂ , W/O BME)	264.92 ± 9.88
Results of the ELISA assay for interleukin-8 (IL-8) performed on placenta- and umbilicus-derived cells as well as human skin fibroblasts. Values are presented here are picograms/million cells, n=2, sem.	
ND: Not Detected	

[0256] Placenta-derived cells were also examined for the production of oxidized LDL receptor, GCP-2 and GROalpha by FACS analysis. Cells tested positive for GCP-2. Oxidized LDL receptor and GRO were not detected by this method.

[0257] Placenta-derived cells were also tested for the production of selected proteins by immunocytochemical analysis. Immediately after isolation (passage 0), cells derived from the human placenta were fixed with 4% paraformaldehyde and exposed to antibodies for six proteins: von Willebrand Factor, CD34, cytokeratin 18, desmin, alpha-smooth muscle actin, and vimentin. Cells stained positive for both alpha-smooth muscle actin and vimentin. This pattern was preserved through passage 11. Only a few cells (<5%) at passage 0 stained positive for cytokeratin 18.

[0258] Cells derived from the human umbilical cord at passage 0 were probed for the production of selected proteins by immunocytochemical analysis. Immediately after isolation (passage 0), cells were fixed with 4% paraformaldehyde and exposed to antibodies for six proteins: von Willebrand Factor, CD34, cytokeratin 18, desmin, alpha-smooth muscle actin, and vimentin. Umbilicus-derived cells were positive for alpha-smooth muscle actin and vimentin, with the staining pattern consistent through passage 11.

[0259] **Summary.** Concordance between gene expression levels measured by microarray and PCR (both real-time and conventional) has been established for four genes: oxidized LDL receptor 1, rennin, reticulon, and IL-8. The expression of these genes was differentially regulated at the mRNA level in the umbilical tissue- and placenta-derived cells, with IL-8 also differentially regulated at the protein level. The presence of oxidized LDL receptor was not detected at the protein level by FACS analysis in cells derived from the placenta. Differential expression of GCP-2 and CXC ligand 3 was not confirmed at the mRNA level, however GCP-2 was detected at the protein level by FACS analysis in the placenta-derived cells. Although this result is not reflected by data originally obtained from the microarray experiment, this may be due to a difference in the sensitivity of the methodologies.

[0260] Immediately after isolation (passage 0), cells derived from the human placenta stained positive for both alpha-smooth muscle actin and vimentin. This pattern was also observed in cells at passage 11. These results suggest that vimentin and alpha-smooth muscle actin expression may be preserved in cells with passaging, in the Growth Medium and under the conditions utilized in these procedures. Cells derived from the human umbilical cord at passage 0 were probed for the expression of alpha-smooth muscle actin and vimentin, and were positive for both. The staining pattern was preserved through passage 11.

EXAMPLE 11

In vitro Immunological Evaluation of Umbilical Cord Tissue and Placenta-Derived Cells

[0261] UTC cells were evaluated *in vitro* for their immunological characteristics in an effort to predict the immunological response, if any, these cells would elicit upon *in vivo* transplantation. UTCs were assayed by flow cytometry for the presence of HLA-DR, HLA-DP, HLA-DQ, CD80, CD86, and B7-H2. These proteins are expressed by antigen-presenting cells (APC) and are required for the direct stimulation of naive CD4⁺ T cells (Abbas & Lichtman, CELLULAR AND MOLECULAR IMMUNOLOGY, 5th Ed. (2003) Saunders, Philadelphia, p. 171). The cell lines were also analyzed by flow cytometry for the expression of HLA-G (Abbas & Lichtman, 2003, *supra*), CD 178 (Couvans, et al., (1999) Journal of Immunological Methods 224, 185-196), and PD-L2 (Abbas & Lichtman, 2003, *supra*; Brown, et. al. (2003) The Journal of Immunology 170, 1257-1266). The expression of these proteins by cells residing in placental tissues is thought to mediate the immuno-privileged status of placental tissues *in utero*. To predict the extent to which placenta- and umbilicus-derived cell lines elicit an immune response *in vivo*, the cell lines were tested in a one-way mixed lymphocyte reaction

(MLR).

Materials and Methods

5 **[0262] Cell culture.** Cells were cultured to confluence in Growth Medium containing penicillin/streptomycin in T75 flasks (Corning, Corning, NY) coated with 2% gelatin (Sigma, St. Louis, MO).

10 **[0263] Antibody Staining.** Cells were washed in phosphate buffered saline (PBS) (Gibco, Carlsbad, CA) and detached with Trypsin/EDTA (Gibco, Carlsbad, MO). Cells were harvested, centrifuged, and re-suspended in 3% (v/v) FBS in PBS at a cell concentration of 1×10^7 per milliliter. Antibody (Table 11-1) was added to one hundred microliters of cell suspension as per manufacturer's specifications and incubated in the dark for 30 minutes at 4°C. After incubation, cells were washed with PBS and centrifuged to remove unbound antibody. Cells were re-suspended in five hundred microliters of PBS and analyzed by flow cytometry using a FACS Calibur instrument (Becton Dickinson, San Jose, CA).

Table 11-1. Antibodies

15	Antibody	Manufacturer	Catalog Number
	HLA-DRDPDQ	BD Pharmingen (San Diego, CA)	555558
	CD80	BD Pharmingen (San Diego, CA)	557227
	CD86	BD Pharmingen (San Diego, CA)	555665
20	B7-H2	BD Pharmingen (San Diego, CA)	552502
	HLA-G	Abcam (Cambridgeshire, UK)	ab 7904-100
	CD 178	Santa Cruz (San Cruz, CA)	sc-19681
	PD-L2	BD Pharmingen (San Diego, CA)	557846
25	Mouse IgG2a	Sigma (St. Louis, MO)	F-6522
	Mouse IgG1kappa	Sigma (St. Louis, MO)	P-4685

30 **[0264] Mixed Lymphocyte Reaction.** Cryopreserved vials of passage 10 umbilicus-derived cells labeled as cell line A and passage 11 placenta-derived cells labeled as cell line B were sent on dry ice to CTBR (Senneville, Quebec) to conduct a mixed lymphocyte reaction using CTBR SOP No. CAC-031. Peripheral blood mononuclear cells (PBMCs) were collected from multiple male and female volunteer donors. Stimulator (donor) allogeneic PBMC, autologous PBMC, and umbilical and placental cell lines were treated with mitomycin C. Autologous and mitomycin C-treated stimulator cells were added to responder (recipient) PBMCs and cultured for 4 days. After incubation, [3 H]thymidine was added to each sample and cultured for 18 hours. Following harvest of the cells, radiolabeled DNA was extracted, and [3 H]-thymidine incorporation was measured using a scintillation counter.

35 **[0265]** The stimulation index for the allogeneic donor (SIAD) was calculated as the mean proliferation of the receiver plus mitomycin C-treated allogeneic donor divided by the baseline proliferation of the receiver. The stimulation index of the UTCs was calculated as the mean proliferation of the receiver plus mitomycin C-treated postpartum cell line divided by the baseline proliferation of the receiver.

40 **Results**

45 **[0266] Mixed lymphocyte reaction - placenta-derived cells.** Seven human volunteer blood donors were screened to identify a single allogeneic donor that would exhibit a robust proliferation response in a mixed lymphocyte reaction with the other six blood donors. This donor was selected as the allogeneic positive control donor. The remaining six blood donors were selected as recipients. The allogeneic positive control donor and placenta-derived cell lines were treated with mitomycin C and cultured in a mixed lymphocyte reaction with the six individual allogeneic receivers. Reactions were performed in triplicate using two cell culture plates with three receivers per plate (Table 11-2). The average stimulation index ranged from 1.3 (plate 2) to 3 (plate 1) and the allogeneic donor positive controls ranged from 46.25 (plate 2) to 279 (plate 1) (Table 11-3).

Table 11-2. Mixed Lymphocyte Reaction Data - Cell Line B (Placenta)

DPM for Proliferation Assay									
Plate ID: Plate1									

(continued)

		Plate ID: Plate1							
5	Analytical	Culture		Replicates					
10	number	System		1	2	3		Mean	SD
15	IM03-7769	Proliferation baseline of receiver		79	119	138		112.0	30.12
		Control of autostimulation (Mitomycin C treated autologous cells)		241	272	175		229.3	49.54
		MLR allogenic donor IM03-7768 (Mitomycin C treated)		23971	22352	20921		22414.7	1525.97
		MLR with cell line (Mitomycin C treated cell type B)		664	559	1090		771.0	281.21
20	SI (donor)							200	
25	SI (cell line)							7	
30	IM03-7770	Proliferation baseline of receiver		206	134	262		200.7	64.17
		Control of autostimulation (Mitomycin C treated autologous cells)		1091	602	524		739.0	307.33
		MLR allogenic donor IM03-7768 (Mitomycin C treated)		45005	43729	44071		44268.3	660.49
		MLR with cell line (Mitomycin C treated cell type B)		533	2582	2376		1830.3	1128.24
35	SI (donor)							221	
40	SI (cell line)							9	
45	IM03-7771	Proliferation baseline of receiver		157	87	128		124.0	35.17
		Control of autostimulation (Mitomycin C treated autologous cells)		293	138	508		313.0	185.81
		MLR allogenic donor IM03-7768 (Mitomycin C treated)		24497	34348	31388		30077.7	5054.53
		MLR with cell line (Mitomycin C treated cell type B)		601	643	a		622.0	29.70
50	SI (donor)							243	
55	SI (cell line)							5	

(continued)

5	Analytical	Culture		Replicates					
10	number	System		1	2	3	Mean	SD	CV
15	IM03-7772	Proliferation baseline of receiver		56	98	51	68.3	25.81	37.8
20		Control of autostimulation (Mitomycin C treated autologous cells)		133	120	213	155.3	50.36	32.4
25		MLR allogenic donor IM03-7768 (Mitomycin C treated)		14222	20076	22168	18822.0	4118.75	21.9
30		MLR with cell line (Mitomycin C treated cell type B)		a	a	a	a	a	a
35	SI (donor)						275		
40	SI (cell line)						a		
45									
50	IM03-7768 (allogenic donor)	Proliferation baseline of receiver		84	242	208	178.0	83.16	46.7
55		Control of autostimulation (Mitomycin treated autologous cells)		361	617	304	427.3	166.71	39.0
30	Cell line type B	Proliferation baseline of receiver		126	124	143	131.0	10.44	8.0
35		Control of autostimulation (Mitomycin treated autologous cells)		822	1075	487	794.7	294.95	37.1
	Plate ID: Plate 2								
40	Analytical number	Culture System		Replicates			Mean	SD	CV
45	IM03-7773	Proliferation baseline of receiver		908	181	330	473.0	384.02	81.2
50		Control of autostimulation (Mitomycin C treated autologous cells)		269	405	572	415.3	151.76	36.5
55		MLR allogenic donor IM03-7768 (Mitomycin C treated)		29151	28691	28315	28719.0	418.70	1.5
		MLR with cell line (Mitomycin C treated cell type B)		567	732	905	734.7	169.02	23.0
	SI (donor)						61		
	SI (cell line)						2		

(continued)

Plate ID: Plate 2								
Analytical number	Culture System		Replicates			Mean	SD	CV
			1	2	3			
IM03-7774	Proliferation baseline of receiver		893	1376	185	818.0	599.03	73.2
	Control of autostimulation (Mitomycin C treated autologous cells)		261	381	568	403.3	154.71	38.4
	MLR allogenic donor							
	IM03-7768 (Mitomycin C treated)		53101	42839	48283	48074.3	5134.18	10.7
IM03-7775	MLR with cell line (Mitomycin C treated cell type B)		515	789	294	532.7	247.97	46.6
	SI (donor)					59		
	SI (cell line)					1		
IM03-7775	Proliferation baseline of receiver		1272	300	544	705.3	505.69	71.7
	Control of autostimulation (Mitomycin C treated autologous cells)		232	199	484	305.0	155.89	51.1
	MLR allogenic donor							
	IM03-7768 (Mitomycin C treated)		23554	10523	28965	21014.0	9479.74	45.1
IM03-7776	MLR with cell line (Mitomycin C treated cell type B)		768	924	563	751.7	181.05	24.1
	SI (donor)					30		
	SI (cell line)					1		
IM03-7776	Proliferation baseline of receiver		1530	137	1046	904.3	707.22	78.2
	Control of autostimulation (Mitomycin C treated autologous cells)		420	218	394	344.0	109.89	31.9
	MLR allogenic donor							
	IM03-7768 (Mitomycin C treated)		28893	32493	34746	32044.0	2952.22	9.2
IM03-7776	MLR with cell line (Mitomycin C treated cell type B)		a	a	a	a	a	a
	SI (donor)					35		
	SI (cell line)					a		

Table 11-3. Average stimulation index of placenta cells and an allogeneic donor in a mixed lymphocyte reaction with six individual allogeneic receivers

Average Stimulation Index		
	Recipient	Placenta
Plate 1 (receivers 1-3)	279	3
Plate 2 (receivers 4-6)	46.25	1.3

[0267] **Mixed lymphocyte reaction - umbilicus-derived cells.** Six human volunteer blood donors were screened to identify a single allogeneic donor that will exhibit a robust proliferation response in a mixed lymphocyte reaction with the other five blood donors. This donor was selected as the allogeneic positive control donor. The remaining five blood donors were selected as recipients. The allogeneic positive control donor and placenta cell lines were mitomycin C-treated and cultured in a mixed lymphocyte reaction with the five individual allogeneic receivers. Reactions were performed in triplicate using two cell culture plates with three receivers per plate (Table 11-4). The average stimulation index ranged from 6.5 (plate 1) to 9 (plate 2) and the allogeneic donor positive controls ranged from 42.75 (plate 1) to 70 (plate 2) (Table 11-5).

Table 11-4. Mixed Lymphocyte Reaction Data- Cell Line A (Umbilicus)

DPM for Proliferation Assay							
Plate ID: Plate1							
Analytical number	Culture System	Replicates			Mean	SD	CV
IM04-2478	Proliferation baseline of receiver	1074	406	391	623.7	390.07	62.5
	Control of autostimulation (Mitomycin C treated autologous cells)	672	510	1402	861.3	475.19	55.2
	MLR allogenic donor IM04-2477 (Mitomycin C treated)	43777	48391	38231	43466.3	5087.12	11.7
	MLR with cell line (Mitomycin C treated cell type A)	2914	5622	6109	4881.7	1721.36	35.3
SI (donor) SI (cell line)					70 8		
IM04-2479	Proliferation baseline of receiver	530	508	527	521.7	11.93	2.3
	Control of autostimulation (Mitomycin C treated autologous cells)	701	567	1111	793.0	283.43	35.7
	MLR allogenic donor IM04-2477 (Mitomycin C treated)	25593	24732	22707	24344.0	1481.61	6.1
	MLR with cell line (Mitomycin C treated cell type A)	5086	3932	1497	3505.0	1832.21	52.3
SI (donor) SI (cell line)					47 7		
IM04-2480	Proliferation baseline of receiver	1192	854	1330	1125.3	244.90	21.8
	Control of autostimulation (Mitomycin C treated autologous cells)	2963	993	2197	2051.0	993.08	48.4
	MLR allogenic donor IM04-2477 (Mitomycin C treated)	25416	29721	23757	26298.0	3078.27	11.7
	MLR with cell line (Mitomycin C treated cell type A)	2596	5076	3426	3699.3	1262.39	34.1

(continued)

DPM for Proliferation Assay Plate ID: Plate1								
Analytical number	Culture System	Replicates			Mean	SD	CV	
		1	2	3				
5	SI (donor) SI (cell line)				23 3			
10	IM04-2481	Proliferation baseline of receiver	695	451	555	567.0	122.44	21.6
		Control of autostimulation (Mitomycin C treated autologous cells)	738	1252	464	818.0	400.04	48.9
		MLR allogenic donor IM04-2477 (Mitomycin C treated)	13177	24885	15444	17835.3	6209.52	34.8
		MLR with cell line (Mitomycin C treated cell type A)	4495	3671	4674	4280.0	534.95	12.5
15	SI (donor) SI (cell line)				31 8			
	Plate 10: Plate 2							
Analytical number	Culture System	Replicates			Mean	SD	CV	
		1	2	3				
20	IM04-2482	Proliferation baseline of receiver	432	533	274	413.0	130.54	31.6
		Control of autostimulation (Mitomycin C treated autologous cells)	1459	633	598	896.7	487.31	54.3
		MLR allogenic donor IM04-2477 (Mitomycin C treated)	24286	30823	31346	28818.3	3933.82	13.7
		MLR with cell line (Mitomycin C treated cell type A)	2762	1502	6723	3662.3	2724.46	74.4
25	SI (donor) SI (cell line)				70 9			
	Plate 10: Plate 2							
Analytical number	Culture System	Replicates			Mean	SD	CV	
		1	2	3				
30	IM04-2477 (allogenic donor)	Proliferation baseline of receiver	312	419	349	360.0	54.34	15.1
		Control of autostimulation (Mitomycin treated autologous cells)	567	604	374	515.0	123.50	24.0
35	Cell line type A	Proliferation baseline of receiver	5101	3735	2973	3936.3	1078.19	27.4
		Control of autostimulation (Mitomycin treated autologous cells)	1924	4570	2153	2882.3	1466.04	50.9

Table 11-5. Average stimulation index of umbilicus-derived cells and an allogeneic donor in a mixed lymphocyte reaction with five individual allogeneic receivers.

Average Stimulation Index		
	Recipient	Umbilicus
Plate 1 (receivers 1-4)	42.75	6.5
Plate 2 (receiver 5)	70	9

[0268] **Antigen presenting cell markers - placenta-derived cells.** Histograms of placenta-derived cells analyzed by flow cytometry show negative expression of HLA-DR, DP, DQ, CD80, CD86, and B7-H2, as noted by fluorescence value consistent with the IgG control, indicating that placental cell lines lack the cell surface molecules required to directly stimulate CD4⁺ T cells.

[0269] **Immunomodulating markers - placenta-derived cells.** Histograms of placenta-derived cells analyzed by flow cytometry show positive expression of PD-L2, as noted by the increased value of fluorescence relative to the IgG control, and negative expression of CD 178 and HLA-G, as noted by fluorescence value consistent with the IgG control.

[0270] **Antigen presenting cell markers - umbilicus-derived cells.** Histograms of umbilicus-derived cells analyzed by flow cytometry show negative expression of HLA-DR, DP, DQ, CD80, CD86, and B7-H2, as noted by fluorescence value consistent with the IgG control, indicating that umbilical cell lines lack the cell surface molecules required to directly stimulate CD4⁺ T cells.

[0271] **Immunomodulating cell markers - umbilicus-derived cells.** Histograms of umbilicus-derived cells analyzed by flow cytometry show positive expression of PD-L2, as noted by the increased value of fluorescence relative to the IgG control, and negative expression of CD 178 and HLA-G, as noted by fluorescence value consistent with the IgG control.

[0272] **Summary.** In the mixed lymphocyte reactions conducted with placenta-derived cell lines, the average stimulation index ranged from 1.3 to 3, and that of the allogeneic positive controls ranged from 46.25 to 279. In the mixed lymphocyte reactions conducted with umbilicus-derived cell lines the average stimulation index ranged from 6.5 to 9, and that of the allogeneic positive controls ranged from 42.75 to 70. Placenta- and umbilicus-derived cell lines were negative for the expression of the stimulating proteins HLA-DR, HLA-DP, HLA-DQ, CD80, CD86, and B7-H2, as measured by flow cytometry. Placenta- and umbilicus-derived cell lines were negative for the expression of immunomodulating proteins HLA-G and CD 178 and positive for the expression of PD-L2, as measured by flow cytometry. Allogeneic donor PBMCs contain antigen-presenting cells expressing HLA-DR, DQ, CD8, CD86, and B7-H2, thereby allowing for the stimulation of naive CD4⁺ T cells. The absence of antigen-presenting cell surface molecules on placenta- and umbilicus-derived cells required for the direct stimulation of naive CD4⁺ T cells and the presence of PD-L2, an immunomodulating protein, may account for the low stimulation index exhibited by these cells in a MLR as compared to allogeneic controls.

EXAMPLE 12

Secretion of Trophic Factors by Umbilical Cord Tissue and Placental-Derived Cells

[0273] The secretion of selected trophic factors from placenta- and umbilicus-derived cells was measured. Factors selected for detection included: (1) those known to have angiogenic activity, such as hepatocyte growth factor (HGF) (Rosen et al. (1997) Ciba Found. Symp. 212:215-26), monocyte chemotactic protein 1 (MCP-1) (Salcedo et al. (2000) Blood 96:34-40), interleukin-8 (IL-8) (Li et al. (2003) J. Immunol. 170:3369-76), keratinocyte growth factor (KGF), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) (Hughes et al. (2004) Ann. Thorac. Surg. 77:812-8), matrix metalloproteinase 1 (TIMP1), angiopoietin 2 (ANG2), platelet derived growth factor (PDGF-bb), thrombopoietin (TPO), heparin-binding epidermal growth factor (HB-EGF), stromal-derived factor 1 alpha (SDF-1 alpha); (2) those known to have neurotrophic/neuroprotective activity, such as brain-derived neurotrophic factor (BDNF) (Cheng et al. (2003) Dev. Biol. 258:319-33), interleukin-6 (IL-6), granulocyte chemotactic protein-2 (GCP-2), transforming growth factor beta2 (TGF β 2); and (3) those known to have chemokine activity, such as macrophage inflammatory protein 1 alpha (MIP1 α), macrophage inflammatory protein 1 beta (MIP1 β), monocyte chemoattractant-1 (MCP-1), Rantes (regulated on activation, normal T cell expressed and secreted), I309, thymus and activation-regulated chemokine (TARC), Eotaxin, macrophage-derived chemokine (MDC), IL-8).

Methods & Materials

[0274] **Cell culture.** Cells from placenta and umbilicus as well as human fibroblasts derived from human neonatal

foreskin were cultured in Growth Medium with penicillin/streptomycin on gelatin-coated T75 flasks. Cells were cryopreserved at passage 11 and stored in liquid nitrogen. After thawing of the cells, Growth Medium was added to the cells followed by transfer to a 15 milliliter centrifuge tube and centrifugation of the cells at 150 x g for 5 minutes. The supernatant was discarded. The cell pellet was resuspended in 4 milliliters Growth Medium, and cells were counted. Cells were seeded at 375,000 cells/75 cm² flask containing 15 milliliters of Growth Medium and cultured for 24 hours. The medium was changed to a serum-free medium (DMEM-low glucose (Gibco), 0.1% (w/v) bovine serum albumin (Sigma), penicillin/streptomycin (Gibco)) for 8 hours. Conditioned serum-free medium was collected at the end of incubation by centrifugation at 14,000 x g for 5 minutes and stored at -20°C. To estimate the number of cells in each flask, cells were washed with PBS and detached using 2 milliliters trypsin/EDTA. Trypsin activity was inhibited by addition of 8 milliliters Growth Medium. Cells were centrifuged at 150 x g for 5 minutes. Supernatant was removed, and cells were resuspended in 1 milliliter Growth Medium. Cell number was estimated using a hemocytometer.

[0275] **ELISA assay.** Cells were grown at 37°C in 5% carbon dioxide and atmospheric oxygen. Placenta-derived cells (batch 101503) also were grown in 5% oxygen or beta-mercaptoethanol (BME). The amount of MCP-1, IL-6, VEGF, SDF-1alpha, GCP-2, IL-8, and TGF-beta 2 produced by each cell sample was measured by an ELISA assay (R&D Systems, Minneapolis, MN). All assays were performed according to the manufacturer's instructions.

[0276] **Searchlight™ multiplexed ELISA assay.** Chemokines (MIP1a, MIP1b, MCP-1, Rantes, I309, TARC, Eotaxin, MDC, IL8), BDNF, and angiogenic factors (HGF, KGF, bFGF, VEGF, TIMP1, ANG2, PDGF-bb, TPO, HB-EGF were measured using SearchLight Proteome Arrays (Pierce Biotechnology Inc.). The Proteome Arrays are multiplexed sandwich ELISAs for the quantitative measurement of two to 16 proteins per well. The arrays are produced by spotting a 2 x 2, 3 x 3, or 4 x 4 pattern of four to 16 different capture antibodies into each well of a 96-well plate. Following a sandwich ELISA procedure, the entire plate is imaged to capture chemiluminescent signal generated at each spot within each well of the plate. The amount of signal generated in each spot is proportional to the amount of target protein in the original standard or sample.

25 Results

[0277] **ELISA assay.** MCP-1 and IL-6 were secreted by placenta- and umbilicus-derived cells and dermal fibroblasts (Table 12-1). SDF-1alpha was secreted by placenta-derived cells cultured in 5% O₂ and by fibroblasts. GCP-2 and IL-8 were secreted by umbilicus-derived cells and by placenta-derived cells cultured in the presence of BME or 5% O₂. GCP-2 also was secreted by human fibroblasts. TGF-beta2 was not detectable by ELISA assay.

Table 12-1. ELISA assay results

(values presented are picograms/milliliter/million cells (n=2, sem)							
	MCP-1	IL-6	VEGF	SDF-1 α	GCP-2	IL-8	TGF- β 2
Fibroblast	17 \pm 1	61 \pm 3	29 \pm 2	19 \pm 1	21 \pm 1	ND	ND
Placenta (042303)	60 \pm 3	41 \pm 2	ND	ND	ND	ND	ND
Umbilicus (022803)	1150 \pm 74	4234 \pm 289	ND	ND	160 \pm 11	2058 \pm 145	ND
Placenta (071003)	125 \pm 16	10 \pm 1	ND	ND	ND	ND	ND
Umbilicus (071003)	2794 \pm 84	1356 \pm 43	ND	ND	2184 \pm 98	2369 \pm 23	ND
Placenta (101503) BME	21 \pm 10	67 \pm 3	ND	ND	44 \pm 9	17 \pm 9	ND
Placenta (101503) 5% O ₂ , W/O BME	77 \pm 16	339 \pm 21	ND	1149 \pm 137	54 \pm 2	265 \pm 10	ND
Key: ND: Not Detected.							

[0278] **Searchlight™ multiplexed ELISA assay.** TIMP1, TPO, KGF, HGF, FGF, HBEGF, BDNF, MIP1b, MCP1, RANTES, I309, TARC, MDC, and IL-8 were secreted from umbilicus-derived cells (Tables 12-2 and 12-3). TIMP1, TPO, KGF, HGF, HBEGF, BDNF, MIP1a, MCP-1, RANTES, TARC, Eotaxin, and IL-8 were secreted from placenta-derived cells (Tables 12-2 and 12-3). No Ang2, VEGF, or PDGF-bb were detected.

Table 12-2. Searchlight™ Multiplexed ELISA assay results

	TIMP1	ANG2	PDGFbb	TPO	KGF	HGF	FGF	VEGF	HBEGF	BDNF
Hfb	19306.3	ND	ND	230.5	5.0	ND	ND	27.9	1.3	ND

(continued)

	TIMP1	ANG2	PDGFbb	TPO	KGF	HGF	FGF	VEGF	HBEGF	BDNF	
5	P1	24299.5	ND	ND	546.6	8.8	16.4	ND	ND	3.81.3	ND
U1	57718.4	ND	ND	1240.0	5.8	559.3	148.7	ND	9.3	165.7	
P3	14176.8	ND	ND	568.7	5.2	10.2	ND	ND	1.9	33.6	
10	U3	21850.0	ND	ND	1134.5	9.0	195.6	30.8	ND	5.4	388.6
Key: hFB (human fibroblasts), P1 (placenta-derived cells (042303)), U1 (umbilicus-derived cells (022803)), P3 (placenta-derived cells(071003)), U3 (umbilicus-derived cells (071003)). ND: Not Detected.											

Table 12-3. Searchlight™ Multiplexed ELISA assay results

	MIP1a	MIP1b	MCP1	RANTES	1309	TARC	Eotaxin	MDC	IL8
hFB	ND	ND	39.6	ND	ND	0.1	ND	ND	204.9
P1	79.5	ND	228.4	4.1	ND	3.8	12.2	ND	413.5
U1	ND	8.0	1694.2	ND	22.4	37.6	ND	18.9	51930.1
P3	ND	ND	102.7	ND	ND	0.4	ND	ND	63.8
U3	ND	5.2	2018.7	41.5	11.6	21.4	ND	4.8	10515.9
Key: hFB (human fibroblasts), P1 (placenta-derived cells (042303)), U1 (umbilicus-derived cells (022803)), P3 (placenta-derived cells (071003)), U3 (umbilicus-derived cells (071003)). ND: Not Detected.									

[0279] Summary. Umbilicus- and placenta-derived cells secreted a number of trophic factors. Some of these trophic factors, such as HGF, bFGF, MCP-1 and IL-8, play important roles in angiogenesis. Other trophic factors, such as BDNF and IL-6, have important roles in neural regeneration.

EXAMPLE 13

Short-Term Neural Differentiation of Umbilical Cord Tissue and Placenta-Derived Cells

[0280] The ability of placenta- and umbilicus-derived cells to differentiate into neural lineage cells was examined.

Materials & Methods

[0281] Isolation and Expansion of Umbilicus- and Placenta-derived Cells. Cells from placental and umbilical tissues were isolated and expanded as described in Example 1.

[0282] Modified Woodbury-Black Protocol. (A) This assay was adapted from an assay originally performed to test the neural induction potential of bone marrow stromal cells (1). Umbilicus-derived cells (022803) P4 and placenta-derived cells (042203) P3 were thawed and culture expanded in Growth Media at 5,000 cells/cm² until sub-confluence (75%) was reached. Cells were then trypsinized and seeded at 6,000 cells per well of a Titretek II glass slide (VWR International, Bristol, CT). As controls, mesenchymal stem cells (P3; 1F2155; Cambrex, Walkersville, MD), osteoblasts (P5; CC2538; Cambrex), adipose-derived cells (Artecel, US6555374 B1) (P6; Donor 2) and neonatal human dermal fibroblasts (P6; CC2509; Cambrex) were also seeded under the same conditions.

[0283] All cells were initially expanded for 4 days in DMEM/F12 medium (Invitrogen, Carlsbad, CA) containing 15% (v/v) fetal bovine serum (FBS; Hyclone, Logan, UT), basic fibroblast growth factor (bFGF; 20 nanograms/milliliter; Peprotech, Rocky Hill, NJ), epidermal growth factor (EGF; 20 nanograms/milliliter; Peprotech) and penicillin/streptomycin (Invitrogen). After four days, cells were rinsed in phosphate-buffered saline (PBS; Invitrogen) and were subsequently cultured in DMEM/F12 medium + 20% (v/v) FBS + penicillin/streptomycin for 24 hours. After 24 hours, cells were rinsed with PBS. Cells were then cultured for 1 - 6 hours in an induction medium which was comprised of DMEM/F12 (serum-free) containing 200 mM butylated hydroxyanisole, 10 µM potassium chloride, 5 milligram/milliliter insulin, 10 µM forskolin, 4 µM valproic acid, and 2 µM hydrocortisone (all chemicals from Sigma, St. Louis, MO). Cells were then fixed in 100% ice-cold methanol and immunocytochemistry was performed (see methods below) to assess human nestin protein expression.

[0284] (B) Derived cells (umbilicus (022803) P11; placenta (042203) P11) and adult human dermal fibroblasts (1F1853, P11) were thawed and culture expanded in Growth Medium at 5,000 cells/cm² until sub-confluence (75%) was reached. Cells were then trypsinized and seeded at similar density as in (A), but onto (1) 24 well tissue culture-treated plates (TCP, Falcon brand, VWR International), (2) TCP wells + 2% (w/v) gelatin adsorbed for 1 hour at room temperature, or (3) TCP wells + 20 µg/milliliter adsorbed mouse laminin (adsorbed for a minimum of 2 hours at 37°C; Invitrogen).

[0285] Exactly as in (A), cells were initially expanded and media switched at the aforementioned timeframes. One set of cultures was fixed, as before, at 5 days and six hours, this time with ice-cold 4% (w/v) paraformaldehyde (Sigma) for 10 minutes at room temperature. In the second set of cultures, medium was removed and switched to Neural Progenitor Expansion medium (NPE) consisting of Neurobasal-A medium (Invitrogen) containing B27 (B27 supplement; Invitrogen), L-glutamine (4 mM), and penicillin/streptomycin (Invitrogen). NPE medium was further supplemented with retinoic acid (RA; 1 µM; Sigma). This medium was removed 4 days later and cultures were fixed with ice-cold 4% (w/v) paraformaldehyde (Sigma) for 10 minutes at room temperature, and stained for nestin, GFAP, and TuJ1 protein expression (see Table 13-1).

15 **Table 13-1.** Summary of Primary Antibodies Used

Antibody	Concentration	Vendor
Rat 401 (nestin)	1:200	Chemicon, Temecula, CA
Human Nestin	1:100	Chemicon
TuJ1 (BIII Tubulin)	1:500	Sigma, St. Louis, MO
GFAP	1:2000	DakoCytomation, Carpinteria, CA
Tyrosine hydroxylase (TH)	1:1000	Chemicon
GABA	1:400	Chemicon
Desmin (mouse)	1:300	Chemicon
alpha - alpha-smooth muscle actin	1:400	Sigma
Human nuclear protein (hNuc)	1:150	Chemicon

[0286] **Two Stage Differentiation Protocol.** Derived cells (umbilicus (042203) P11, placenta (022803) P11), adult human dermal fibroblasts (P11; 1F1853; Cambrex) were thawed and culture expanded in Growth Medium at 5,000 cells/cm² until sub-confluence (75%) was reached. Cells were then trypsinized and seeded at 2,000 cells/cm², but onto 24 well plates coated with laminin (BD Biosciences, Franklin Lakes, NJ) in the presence of NPE media supplemented with bFGF (20 nanograms/milliliter; Peprotech, Rocky Hill, NJ) and EGF (20 nanograms/milliliter; Peprotech) [whole media composition further referred to as NPE + F + E]. At the same time, adult rat neural progenitors isolated from hippocampus (P4; 062603) were also plated onto 24 well laminin-coated plates in NPE + F + E media. All cultures were maintained in such conditions for a period of 6 days (cells were fed once during that time) at which time media was switched to the differentiation conditions listed in Table N1-2 for an additional period of 7 days. Cultures were fixed with ice-cold 4% (w/v) paraformaldehyde (Sigma) for 10 minutes at room temperature, and stained for human or rat nestin, GFAP, and TuJ1 protein expression.

40 **Table 13-2.** Summary of Conditions for Two-Stage Differentiation Protocol

COND. #	PRE-DIFFERENTIATION	A	B
		2 nd STAGE DIFF	
1	NPE + F (20 ng/ml) + E (20 ng/ml)	NPE + SHH (200 ng/ml) + F8 (100 ng/ml)	
2	NPE + F (20 ng/ml) + E (20 ng/ml)	NPE + SHH (200 ng/ml) + F8 (100 ng/ml) + RA (1 µM)	
3	NPE + F (20 ng/ml) + E (20 ng/ml)	NPE + RA (1 µM)	
4	NPE + F (20 ng/ml) + E (20 ng/ml)	NPE + F (20 ng/ml) + E (20 ng/ml)	
5	NPE + F (20 ng/ml) + E (20 ng/ml)	Growth Medium	
6	NPE + F (20 ng/ml) + E (20 ng/ml)	Condition 1B + MP52 (20 ng/ml)	
7	NPE + F (20 ng/ml) + E (20 ng/ml)	Condition 1B + BMP7 (20 ng/ml)	
8	NPE + F (20 ng/ml) + E (20 ng/ml)	Condition 1B + GDNF (20 ng/ml)	
9	NPE + F (20 ng/ml) + E (20 ng/ml)	Condition 2B + MP52 (20 ng/ml)	

(continued)

A		B
COND. #	PRE-DIFFERENTIATION	2 nd STAGE DIFF
10	NPE + F (20 ng/ml) + E (20 ng/ml)	Condition 2B + BMP7 (20 ng/ml)
11	NPE + F (20 ng/ml) + E (20 ng/ml)	Condition 2B + GDNF (20 ng/ml)
12	NPE + F (20 ng/ml) + E (20 ng/ml)	Condition 3B + MP52 (20 ng/ml)
13	NPE + F (20 ng/ml) + E (20 ng/ml)	Condition 3B + BMP7 (20 ng/ml)
14	NPE + F (20 ng/ml) + E (20 ng/ml)	Condition 3B + GDNF (20 ng/ml)
15	NPE + F (20 ng/ml) + E (20 ng/ml)	NPE + MP52 (20 ng/ml)
16	NPE + F (20 ng/ml) + E (20 ng/ml)	NPE + BMP7 (20 ng/ml)
17	NPE + F (20 ng/ml) + E (20 ng/ml)	NPE + GDNF (20 ng/ml)

[0287] Multiple growth factor protocol. Umbilicus-derived cells (P11; (042203)) were thawed and culture expanded in Growth Medium at 5,000 cells/cm² until sub-confluence (75%) was reached. Cells were then trypsinized and seeded at 2,000 cells/cm², onto 24 well laminin-coated plates (BD Biosciences) in the presence of NPE + F (20 nanograms/milliliter) + E (20 nanograms/milliliter). In addition, some wells contained NPE + F + E + 2% FBS or 10% FBS. After four days of "pre-differentiation" conditions, all media were removed and samples were switched to NPE medium supplemented with sonic hedgehog (SHH; 200 nanograms/milliliter; Sigma, St. Louis, MO), FGF8 (100 nanograms/milliliter; Peprotech), BDNF (40 nanograms/milliliter; Sigma), GDNF (20 nanograms/milliliter; Sigma), and retinoic acid (1 μ M; Sigma). Seven days post medium change, cultures were fixed with ice-cold 4% (w/v) paraformaldehyde (Sigma) for 10 minutes at room temperature, and stained for human nestin, GFAP, TuJ1, desmin, and alpha-smooth muscle actin expression.

[0288] Neural progenitor co-culture protocol. Adult rat hippocampal progenitors (062603) were plated as neurospheres or single cells (10,000 cells/well) onto laminin-coated 24 well dishes (BD Biosciences) in NPE + F (20 nanograms/milliliter) + E (20 nanograms/milliliter).

[0289] Separately, umbilicus-derived cells (042203) P11 and placenta-derived cells (022803) P11 were thawed and culture expanded in NPE + F (20 nanograms/milliliter) + E (20 nanograms/milliliter) at 5,000 cells/cm² for a period of 48 hours. Cells were then trypsinized and seeded at 2,500 cells/well onto existing cultures of neural progenitors. At that time, existing medium was exchanged for fresh medium. Four days later, cultures were fixed with ice-cold 4% (w/v) paraformaldehyde (Sigma) for 10 minutes at room temperature, and stained for human nuclear protein (hNuc; Chemicon) (Table NU1-1 above) to identify the umbilical cord tissue- and placenta-derived cells.

[0290] Immunocytochemistry. Immunocytochemistry was performed using the antibodies listed in Table NU1-1. Cultures were washed with phosphate-buffered saline (PBS) and exposed to a protein blocking solution containing PBS, 4% (v/v) goat serum (Chemicon, Temecula, CA), and 0.3% (v/v) Triton (Triton X-100; Sigma) for 30 minutes to access intracellular antigens. Primary antibodies, diluted in blocking solution, were then applied to the cultures for a period of 1 hour at room temperature. Next, primary antibodies solutions were removed and cultures washed with PBS prior to application of secondary antibody solutions (1 hour at room temperature) containing blocking solution along with goat anti-mouse IgG - Texas Red (1:250; Molecular Probes, Eugene, OR) and goat anti-rabbit IgG - Alexa 488 (1:250; Molecular Probes). Cultures were then washed and 10 micromolar DAPI (Molecular Probes) applied for 10 minutes to visualize cell nuclei.

[0291] Following immunostaining, fluorescence was visualized using the appropriate fluorescence filter on an Olympus inverted epi-fluorescent microscope (Olympus, Melville, NY). In all cases, positive staining represented fluorescence signal above control staining where the entire procedure outlined above was followed with the exception of application of a primary antibody solution. Representative images were captured using a digital color videocamera and ImagePro software (Media Cybernetics, Carlsbad, CA). For triple-stained samples, each image was taken using only one emission filter at a time. Layered montages were then prepared using Adobe Photoshop software (Adobe, San Jose, CA).

Results

[0292] Woodbury-Black protocol. (Woodbury, D. et al. (2000). J Neurosci. Research. 61(4): 364-70). (A) Upon incubation in this neural induction composition, all cell types transformed into cells with bipolar morphologies and extended processes. Other larger non-bipolar morphologies were also observed. Furthermore, the induced cell populations stained

positively for nestin, a marker of multipotent neural stem and progenitor cells.

[0293] (B) When repeated on tissue culture plastic (TCP) dishes, nestin expression was not observed unless laminin was pre-adsorbed to the culture surface. To further assess whether nestin-expressing cells could then go on to generate mature neurons, the umbilical cord tissue derived-cells, placenta-derived cells and fibroblasts were exposed to NPE + RA (1 μ M), a media composition known to induce the differentiation of neural stem and progenitor cells into such cells (Jang, Y.K. et al. (2004). *J. Neurosci. Research.* 75(4): 573-84; Jones-Villeneuve, E.M. et al. (1983). *Mol Cel Biol.* 3(12): 2271-9; Mayer-Proschel, M. et al. (1997). *Neuron.* 19(4): 773-85). Cells were stained for TuJ1, a marker for immature and mature neurons, GFAP, a marker of astrocytes, and nestin. Under no conditions was TuJ1 detected, nor were cells with neuronal morphology observed, suggesting that neurons were not generated in the short term. Furthermore, nestin and GFAP were no longer expressed by umbilical and placental-derived cells, as determined by immunocytochemistry.

[0294] **Two-stage differentiation.** Umbilicus and placenta isolates (as well as human fibroblasts and rodent neural progenitors as negative and positive control cell types, respectively) were plated on laminin (neural promoting)-coated dishes and exposed to 13 different growth conditions (and two control conditions) known to promote differentiation of neural progenitors into neurons and astrocytes. In addition, two conditions were added to examine the influence of GDF5, and BMP7 on cell differentiation. Generally, a two-step differentiation approach was taken, where the cells were first placed in neural progenitor expansion conditions for a period of 6 days, followed by full differentiation conditions for 7 days. Morphologically, both umbilicus- and placenta-derived cells exhibited fundamental changes in cell morphology throughout the time-course of this procedure. However, neuronal or astrocytic-shaped cells were not observed except for in control, neural progenitor-plated conditions. Immunocytochemistry, negative for human nestin, TuJ1, and GFAP confirmed the morphological observations.

[0295] **Multiple growth factors.** Following one week's exposure to a variety of neural differentiation agents, cells were stained for markers indicative of neural progenitors (human nestin), neurons (TuJ1), and astrocytes (GFAP). Cells grown in the first stage in non-serum containing media had different morphologies than those cells in serum containing (2% or 10%) media, indicating potential neural differentiation. Specifically, following a two step procedure of exposing umbilicus-derived cells to EGF and bFGF, followed by SHH, FGF8, GDNF, BDNF, and retinoic acid, cells showed long extended processes similar to the morphology of cultured astrocytes. When 2% FBS or 10% FBS was included in the first stage of differentiation, cell number was increased and cell morphology was unchanged from control cultures at high density. Potential neural differentiation was not evidenced by immunocytochemical analysis for human nestin, TuJ1, or GFAP.

[0296] **Neural progenitor and UTC co-culture.** Umbilical and placental-derived cells were plated onto cultures of rat neural progenitors seeded two days earlier in neural expansion conditions (NPE + F + E). While visual confirmation of plated UTCs and placenta derived cells proved that these cells were plated as single cells, human-specific nuclear staining (hNuc) 4 days post-plating (6 days total) showed that they tended to ball up and avoid contact with the neural progenitors. Furthermore, where UTCs attached, these cells spread out and appeared to be innervated by differentiated neurons that were of rat origin, suggesting that the UTCs may have differentiated into muscle cells. This observation was based upon morphology under phase contrast microscopy. Another observation was that typically large cell bodies (larger than neural progenitors) possessed morphologies resembling neural progenitors, with thin processes spanning out in multiple directions. HNuc staining (found in one half of the cell's nucleus) suggested that in some cases these human cells may have fused with rat progenitors and assumed their phenotype. Control wells containing only neural progenitors had fewer total progenitors and apparent differentiated cells than did co-culture wells containing umbilicus or placenta derived cells, further indicating that both umbilicus- and placenta-derived cells influenced the differentiation and behavior of neural progenitors, either by release of chemokines and cytokines, or by contact-mediated effects.

[0297] **Summary.** Multiple protocols were conducted to determine the short term potential of UTCs and placenta derived cells to differentiate into neural lineage cells. These included phase contrast imaging of morphology in combination with immunocytochemistry for nestin, TuJ1, and GFAP, proteins associated with multipotent neural stem and progenitor cells, immature and mature neurons, and astrocytes, respectively. Evidence was observed to suggest that neural differentiation occurred in certain instances in these short-term protocols.

[0298] Several notable observations were made in co-cultures of UTCs and placenta derived cells with neural progenitors. This approach, using human UTCs and placenta derived cells along with a xenogeneic cell type allowed for absolute determination of the origin of each cell in these cultures. First, some cells were observed in these cultures where the cell cytoplasm was enlarged, with neurite-like processes extending away from the cell body, yet only half of the body labeled with hNuc protein. Those cells may have been human UTCs and placenta derived cells that had differentiated into neural lineage cells or they may have been UTCs and placenta derived cells that had fused with neural progenitors. Second, it appeared that neural progenitors extended neurites to UTCs and placenta derived cells in a way that indicates the progenitors differentiated into neurons and innervated the UTCs and placenta derived cells. Third, cultures of neural progenitors and UTCs and placenta derived cells had more cells of rat origin and larger amounts of differentiation than control cultures of neural progenitors alone, further indicating that plated UTCs and placenta derived cells provided soluble factors and or contact-dependent mechanisms that stimulated neural progenitor survival, prolif-

eration, and/or differentiation.

EXAMPLE 14

5 Long-Term Neural Differentiation of Umbilical Cord Tissue and Placenta-Derived Cells

[0299] The ability of umbilicus and placenta-derived cells to undergo long-term differentiation into neural lineage cells was evaluated.

10 Materials & Methods

[0300] **Isolation and Expansion of Cells.** Cells were isolated and expanded as described in previous Examples.

[0301] **Cell Thaw and Plating.** Frozen aliquots of cells (umbilicus (022803) P11; (042203) P11; (071003) P12; placenta (101503) P7) previously grown in Growth Medium were thawed and plated at 5,000 cells/cm² in T-75 flasks coated with laminin (BD, Franklin Lakes, NJ) in Neurobasal-A medium (Invitrogen, Carlsbad, CA) containing B27 (B27 supplement, Invitrogen), L-glutamine (4 mM), and Penicillin/Streptomycin (10 milliliters), the combination of which is herein referred to as Neural Progenitor Expansion (NPE) media. NPE media was further supplemented with bFGF (20 nanograms/milliliter, Peprotech, Rocky Hill, NJ) and EGF (20 nanograms/milliliter, Peprotech, Rocky Hill, NJ), herein referred to as NPE + bFGF + EGF.

[0302] **Control Cell Plating.** In addition, adult human dermal fibroblasts (P11, Cambrex, Walkersville, MD) and mesenchymal stem cells (P5, Cambrex) were thawed and plated at the same cell seeding density on laminin-coated T-75 flasks in NPE + bFGF + EGF. As a further control, fibroblasts, umbilicus, and placenta cells were grown in Growth Medium for the period specified for all cultures.

[0303] **Cell Expansion.** Media from all cultures were replaced with fresh media once a week and cells observed for expansion. In general, each culture was passaged one time over a period of one month because of limited growth in NPE + bFGF + EGF.

[0304] **Immunocytochemistry.** After a period of one month, all flasks were fixed with cold 4% (w/v) paraformaldehyde (Sigma) for 10 minutes at room temperature. Immunocytochemistry was performed using antibodies directed against TuJ1 (BIII Tubulin; 1:500; Sigma, St. Louis, MO) and GFAP (glial fibrillary acidic protein; 1:2000; DakoCytomation, Carpinteria, CA). Briefly, cultures were washed with phosphate-buffered saline (PBS) and exposed to a protein blocking solution containing PBS, 4% (v/v) goat serum (Chemicon, Temecula, CA), and 0.3% (v/v) Triton (Triton X-100; Sigma) for 30 minutes to access intracellular antigens. Primary antibodies, diluted in blocking solution, were then applied to the cultures for a period of 1 hour at room temperature. Next, primary antibodies solutions were removed and cultures washed with PBS prior to application of secondary antibody solutions (1 hour at room temperature) containing block along with goat anti-mouse IgG - Texas Red (1:250; Molecular Probes, Eugene, OR) and goat anti-rabbit IgG - Alexa 488 (1:250; Molecular Probes). Cultures were then washed and 10 micromolar DAPI (Molecular Probes) applied for 10 minutes to visualize cell nuclei.

[0305] Following immunostaining, fluorescence was visualized using the appropriate fluorescence filter on an Olympus inverted epi-fluorescent microscope (Olympus, Melville, NY). In all cases, positive staining represented fluorescence signal above control staining where the entire procedure outlined above was followed with the exception of application of a primary antibody solution. Representative images were captured using a digital color videocamera and ImagePro software (Media Cybernetics, Carlsbad, CA). For triple-stained samples, each image was taken using only one emission filter at a time. Layered montages were then prepared using Adobe Photoshop software (Adobe, San Jose, CA).

45 **Table 14-1. Summary of Primary Antibodies Used**

Antibody	Concentration	Vendor
TuJ1 (BIII Tubulin)	1:500	Sigma, St. Louis, MO
GFAP	1:2000	DakoCytomation, Carpinteria, CA

50 Results

[0306] **NPE + bFGF + EGF media slows proliferation of UTCs and alters their morphology.** Immediately following plating, a subset of cells attached to the culture flasks coated with laminin. This may have been due to cell death as a function of the freeze/thaw process or because of the new growth conditions. Cells that did attach adopted morphologies different from those observed in Growth Media.

[0307] Upon confluence, cultures were passaged and observed for growth. Very little expansion took place of those

cells that survived passage. At this point, very small cells with no spread morphology and with phase-bright characteristics began to appear in cultures of umbilicus-derived cells. These areas of the flask were followed over time. From these small cells, bifurcating processes emerged with varicosities along their lengths, features very similar to previously described PSA-NCAM+ neuronal progenitors and TuJ1 + immature neurons derived from brain and spinal cord (Mayer-Proschel, M. et al. (1997). *Neuron*. 19(4): 773-85; Yang, H. et al. (2000). *PNAS*. 97(24): 13366-71). With time, these cells became more numerous, yet still were only found in clones.

5 **[0308] Clones of umbilicus-derived cells express neuronal proteins.** Cultures were fixed at one month post-thawing/plating and stained for the neuronal protein TuJ1 and GFAP, an intermediate filament found in astrocytes. While all control cultures grown in Growth Medium and human fibroblasts and MSCs grown in NPE + bFGF + EGF medium 10 were found to be TuJ1-/GFAP-, TuJ1 was detected in the umbilicus and placenta derived cells. Expression was observed in cells with and without neuronal-like morphologies. No expression of GFAP was observed in either culture. The percentage of cells expressing TuJ1 with neuronal-like morphologies was less than or equal to 1% of the total population (n = 3 umbilicus-derived cell isolates tested). While not quantified, the percentage of TuJ1 + cells without neuronal 15 morphologies was higher in umbilicus-derived cell cultures than placenta-derived cell cultures. These results appeared specific as age-matched controls in Growth Medium did not express TuJ1.

10 **[0309] Summary.** Methods for generating differentiated neurons (based on TuJ1 expression and neuronal morphology) 15 from umbilicus-derived cells were developed. While expression for TuJ1 was not examined earlier than one month *in vitro*, it is clear that at least a small population of umbilicus-derived cells can give rise to neurons either through default 20 differentiation or through long-term induction following one month's exposure to a minimal media supplemented with L-glutamine, basic FGF, and EGF.

EXAMPLE 15

Trophic Factors for Neural Progenitor Support

25 **[0310]** The influence of umbilicus- and placenta-derived cells on adult neural stem and progenitor cell survival and differentiation through non-contact dependent (trophic) mechanisms was examined.

Materials & Methods

30 **[0311] Adult neural stem and progenitor cell isolation.** Fisher 344 adult rats were sacrificed by CO₂ asphyxiation followed by cervical dislocation. Whole brains were removed intact using bone rongeurs and hippocampus tissue dissected based on coronal incisions posterior to the motor and somatosensory regions of the brain (Paxinos, G. & Watson, C. 1997. *The Rat Brain in Stereotaxic Coordinates*). Tissue was washed in Neurobasal-A medium (Invitrogen, Carlsbad, CA) containing B27 (B27 supplement; Invitrogen), L-glutamine (4mM; Invitrogen), and penicillin/streptomycin (Invitrogen), the combination of which is herein referred to as Neural Progenitor Expansion (NPE) medium. NPE medium was further supplemented with bFGF (20 nanograms/milliliter, Peprotech, Rocky Hill, NJ) and EGF (20 nanograms/milliliter, Peprotech, Rocky Hill, NJ), herein referred to as NPE + bFGF + EGF.

35 **[0312]** Following wash, the overlying meninges were removed, and the tissue minced with a scalpel. Minced tissue was collected and trypsin/EDTA (Invitrogen) added as 75% of the total volume. DNase (100 microliters per 8 milliliters total volume, Sigma, St. Louis, MO) was also added. Next, the tissue/media was sequentially passed through an 18 gauge needle, 20 gauge needle, and finally a 25 gauge needle one time each (all needles from Becton Dickinson, Franklin Lakes, NJ). The mixture was centrifuged for 3 minutes at 250 g. Supernatant was removed, fresh NPE + bFGF + EGF was added and the pellet resuspended. The resultant cell suspension was passed through a 40 micrometer cell strainer (Becton Dickinson), plated on laminin-coated T-75 flasks (Becton Dickinson) or low cluster 24-well plates (Becton Dickinson), and grown in NPE + bFGF + EGF media until sufficient cell numbers were obtained for the studies outlined.

40 **[0313] Cell plating.** Derived cells (umbilicus (022803) P12, (042103) P12, (071003) P12; placenta (042203) P12) previously grown in Growth Medium were plated at 5,000 cells / transwell insert (sized for 24 well plate) and grown for a period of one week in Growth Medium in inserts to achieve confluence.

45 **[0314] Adult neural progenitor plating.** Neural progenitors, grown as neurospheres or as single cells, were seeded onto laminin-coated 24 well plates at an approximate density of 2,000 cells / well in NPE + bFGF + EGF for a period of one day to promote cellular attachment. One day later, transwell inserts containing derived cells were added according to the following scheme:

50

- (1) Transwell (umbilicus-derived cells in Growth Media, 200 microliters) + neural progenitors (NPE + bFGF + EGF, 1 milliliter)
- (2) Transwell (placenta-derived cells in Growth Media, 200 microliters) + neural progenitors (NPE + bFGF + EGF, 1 milliliter)

(3) Transwell (adult human dermal fibroblasts [1F1853; Cambrex, Walkersville, MD] P12 in Growth Media, 200 microliters) + neural progenitors (NPE + bFGF + EGF, 1 milliliter)
 (4) Control: neural progenitors alone (NPE + bFGF + EGF, 1 milliliter)
 (5) Control: neural progenitors alone (NPE only, 1 milliliter)

5 [0315] **Immunocytochemistry.** After 7 days in co-culture, all conditions were fixed with cold 4% (w/v) paraformaldehyde (Sigma) for a period of 10 minutes at room temperature. Immunocytochemistry was performed using antibodies directed against the epitopes listed in Table 15-1. Briefly, cultures were washed with phosphate-buffered saline (PBS) and exposed to a protein blocking solution containing PBS, 4% (v/v) goat serum (Chemicon, Temecula, CA), and 0.3% (v/v) Triton (Triton X-100; Sigma) for 30 minutes to access intracellular antigens. Primary antibodies, diluted in blocking solution, were then applied to the cultures for a period of 1 hour at room temperature. Next, primary antibodies solutions were removed and cultures washed with PBS prior to application of secondary antibody solutions (1 hour at room temperature) containing blocking solution along with goat anti-mouse IgG - Texas Red (1:250; Molecular Probes, Eugene, OR) and goat anti-rabbit IgG - Alexa 488 (1:250; Molecular Probes). Cultures were then washed and 10 micromolar DAPI (Molecular Probes) applied for 10 minutes to visualize cell nuclei.

10 [0316] Following immunostaining, fluorescence was visualized using the appropriate fluorescence filter on an Olympus inverted epi-fluorescent microscope (Olympus, Melville, NY). In all cases, positive staining represented fluorescence signal above control staining where the entire procedure outlined above was followed with the exception of application of a primary antibody solution. Representative images were captured using a digital color videocamera and ImagePro 15 software (Media Cybernetics, Carlsbad, CA). For triple-stained samples, each image was taken using only one emission filter at a time. Layered montages were then prepared using Adobe Photoshop software (Adobe, San Jose, CA).

20 **Table 15-1. Summary of Primary Antibodies Used**

25 Antibody	Concentration	Vendor
Rat 401 (nestin)	1:200	Chemicon, Temecula, CA
TuJ1 (BIII Tubulin)	1:500	Sigma, St. Louis, MO
Tyrosine hydroxylase (TH)	1:1000	Chemicon
GABA	1:400	Chemicon
30 GFAP	1:2000	DakoCytomation, Carpinteria, CA
Myelin Basic Protein (MBP)	1:400	Chemicon

35 [0317] **Quantitative analysis of neural progenitor differentiation.** Quantification of hippocampal neural progenitor differentiation was examined. A minimum of 1000 cells were counted per condition or if less, the total number of cells observed in that condition. The percentage of cells positive for a given stain was assessed by dividing the number of positive cells by the total number of cells as determined by DAPI (nuclear) staining.

40 [0318] **Mass spectrometry analysis & 2D gel electrophoresis.** In order to identify unique, secreted factors as a result of co-culture, conditioned media samples taken prior to culture fixation were frozen down at -80°C overnight. Samples were then applied to ultrafiltration spin devices (MW cutoff 30 kD). Retentate was applied to immunoaffinity chromatography (anti-Hu-albumin; IgY) (immunoaffinity did not remove albumin from the samples). Filtrate was analyzed by MALDI. The pass through was applied to Cibachron Blue affinity chromatography. Samples were analyzed by SDS-PAGE and 2D gel electrophoresis.

45 **Results**

50 [0319] **Derived cell co-culture stimulates adult neural progenitor differentiation.** Following culture with umbilicus- or placenta-derived cells, co-cultured neural progenitor cells derived from adult rat hippocampus exhibited significant differentiation along all three major lineages in the central nervous system. This effect was clearly observed after five days in co-culture, with numerous cells elaborating complex processes and losing their phase bright features characteristic of dividing progenitor cells. Conversely, neural progenitors grown alone in the absence of bFGF and EGF appeared unhealthy and survival was limited.

55 [0320] After completion of the procedure, cultures were stained for markers indicative of undifferentiated stem and progenitor cells (nestin), immature and mature neurons (TuJ1), astrocytes (GFAP), and mature oligodendrocytes (MBP). Differentiation along all three lineages was confirmed while control conditions did not exhibit significant differentiation as evidenced by retention of nestin-positive staining amongst the majority of cells. While both umbilicus- and placenta-derived cells induced cell differentiation, the degree of differentiation for all three lineages was less in co-cultures with placenta-derived cells than in co-cultures with umbilicus-derived cells.

[0321] The percentage of differentiated neural progenitors following co-culture with umbilicus-derived cells was quantified (Table 15-2). Umbilicus-derived cells significantly enhanced the number of mature oligodendrocytes (MBP) (24.0% vs 0% in both control conditions). Furthermore, co-culture enhanced the number of GFAP+ astrocytes and TuJ1 + neurons in culture (47.2% and 8.7% respectively). These results were confirmed by nestin staining indicating that progenitor status was lost following co-culture (13.4% vs 71.4% in control condition 4).

[0322] Though differentiation also appeared to be influenced by adult human fibroblasts, such cells were not able to promote the differentiation of mature oligodendrocytes nor were they able to generate an appreciable quantity of neurons. Though not quantified, fibroblasts did, however, appear to enhance the survival of neural progenitors.

10 **Table 15-2.** Quantification of progenitor differentiation in control vs transwell co-culture with umbilical-derived cells (E=EGF, F=bFGF)

Antibody	F+E / Umb [Cond. 1]	F+E/F+E [Cond. 4]	F+E/removed [Cond. 5]
TuJ1	8.7 %	2.3 %	3.6%
GFAP	47.2 %	30.2 %	10.9 %
MBP	23.0 %	0%	0%
Nestin	13.4 %	71.4 %	39.4 %

20 [0323] **Identification of unique compounds.** Conditioned media from umbilicus- and placenta- derived co-cultures, along with the appropriate controls (NPE media \pm 1.7 % serum, media from co-culture with fibroblasts), were examined for differences. Potentially unique compounds were identified and excised from their respective 2D gels.

25 [0324] **Summary.** Co-culture of adult neural progenitor cells with umbilicus or placenta derived cells results in differentiation of those cells. Results presented in this example indicate that the differentiation of adult neural progenitor cells following co-culture with umbilicus-derived cells is particularly profound. Specifically, a significant percentage of mature oligodendrocytes was generated in co-cultures of umbilicus-derived cells. In view of the lack of contact between the umbilicus-derived cells and the neural progenitors, this result appears to be a function of soluble factors released from the umbilicus-derived cells (trophic effect).

30 [0325] **Several other observations were made.** First, there were very few cells in the control condition where EGF and bFGF were removed. Most cells died and on average, there were about 100 cells or fewer per well. Second, it is to be expected that there would be very little differentiation in the control condition where EGF and bFGF was retained in the medium throughout, since this is normally an expansion medium. While approximately 70% of the cells were observed to retain their progenitor status (nestin+), about 30% were GFAP+ (indicative of astrocytes). This may be due to the fact that such significant expansion occurred throughout the course of the procedure that contact between progenitors induced this differentiation (Song, H. et al. 2002. Nature 417(6884): 39-44).

EXAMPLE 16

Transplantation of Umbilicus- and Placenta-Derived Cells

40 [0326] Cells derived from the umbilicus and placenta are useful for regenerative therapies. The tissue produced by umbilical cord tissue and placenta-derived cells transplanted into SCID mice with a biodegradable material was evaluated. The materials evaluated were Vicryl non-woven, 35/65 PCL/PGA foam, and RAD 16 self-assembling peptide hydrogel.

Methods & Materials

[0327] **Cell Culture.** Placenta- and umbilicus-derived cells were grown in Growth Medium (DMEM-low glucose (Gibco, Carlsbad CA), 15% (v/v) fetal bovine serum (Cat. #SH30070.03; Hyclone, Logan, UT), 0.001% (v/v) betamercaptoethanol (Sigma, St. Louis, MO), penicillin/streptomycin (Gibco)) in a gelatin-coated flasks.

50 [0328] **Sample Preparation.** One million viable cells were seeded in 15 microliters Growth Medium onto 5 mm diameter, 2.25 mm thick Vicryl non-woven scaffolds (64.33 milligrams/cc; Lot#3547-47-1) or 5 mm diameter 35/65 PCL/PGA foam (Lot# 3415-53). Cells were allowed to attach for two hours before adding more Growth Medium to cover the scaffolds. Cells were grown on scaffolds overnight. Scaffolds without cells were also incubated in medium.

55 [0329] RAD16 self-assembling peptides (3D Matrix, Cambridge, MA under a material transfer agreement) was obtained as a sterile 1 % (w/v) solution in water, which was mixed 1:1 with 1×10^6 cells in 10% (w/v) sucrose (Sigma, St Louis, MO), 10 mM HEPES in Dulbecco's modified medium (DMEM; Gibco) immediately before use. The final concentration of cells in RAD16 hydrogel was 1×10^6 cells/100 microliters.

TEST MATERIAL (N=4/Rx)

- 5 1. Vicryl non-woven + 1 x 10⁶ umbilicus-derived cells
2. 35/65 PCL/PGA foam + 1 x 10⁶ umbilicus-derived cells
3. RAD 16 self-assembling peptide + 1 x 10⁶ umbilicus-derived cells
4. Vicryl non-woven + 1 x 10⁶ placenta-derived cells
5. 35/65 PCL/PGA foam + 1 x 10⁶ placenta-derived cells
6. RAD 16 self-assembling peptide + 1 x 10⁶ placenta-derived cells
- 10 7. 35/65 PCL/PGA foam
8. Vicryl non-woven

15 **[0330] Animal Preparation.** The animals were handled and maintained in accordance with the current requirements of the Animal Welfare Act. Compliance with the above Public Laws were accomplished by adhering to the Animal Welfare regulations (9 CFR) and conforming to the current standards promulgated in the Guide for the Care and Use of Laboratory Animals, 7th edition.

20 **[0331] Mice (Mus Musculus)/Fox Chase SCID/Male (Harlan Sprague Dawley, Inc., Indianapolis, Indiana), 5 weeks of age.** All handling of the SCID mice took place under a hood. The mice were individually weighed and anesthetized with an intraperitoneal injection of a mixture of 60 milligrams/kg KETASET (ketamine hydrochloride, Aveco Co., Inc., Fort Dodge, Iowa) and 10 milligrams/kg ROMPUN (xylazine, Mobay Corp., Shawnee, Kansas) and saline. After induction of anesthesia, the entire back of the animal from the dorsal cervical area to the dorsal lumbosacral area was clipped free of hair using electric animal clippers. The area was then scrubbed with chlorhexidine diacetate, rinsed with alcohol, dried, and painted with an aqueous iodophor solution of 1% available iodine. Ophthalmic ointment was applied to the eyes to prevent drying of the tissue during the anesthetic period.

25 **[0332] Subcutaneous Implantation Technique.** Four skin incisions, each approximately 1.0 cm in length, were made on the dorsum of the mice. Two cranial sites were located transversely over the dorsal lateral thoracic region, about 5-mm caudal to the palpated inferior edge of the scapula, with one to the left and one to the right of the vertebral column. Another two were placed transversely over the gluteal muscle area at the caudal sacro-lumbar level, about 5-mm caudal to the palpated iliac crest, with one on either side of the midline. Implants were randomly placed in these sites in accordance with the experimental design. The skin was separated from the underlying connective tissue to make a small pocket and the implant placed (or injected for RAD16) about 1-cm caudal to the incision. The appropriate test material was implanted into the subcutaneous space. The skin incision was closed with metal clips.

30 **[0333] Animal Housing.** Mice were individually housed in microisolator cages throughout the course of the study within a temperature range of 64°F - 79°F and relative humidity of 30% to 70%, and maintained on an approximate 12 hour light/12 hour dark cycle. The temperature and relative humidity were maintained within the stated ranges to the greatest extent possible. Diet consisted of Irradiated Pico Mouse Chow 5058 (Purina Co.) and water fed *ad libitum*.

35 **[0334] Mice** were euthanized at their designated intervals by carbon dioxide inhalation. The subcutaneous implantation sites with their overlying skin were excised and frozen for histology.

40 **[0335] Histology.** Excised skin with implant was fixed with 10% neutral buffered formalin (Richard-Allan Kalamazoo, MI). Samples with overlying and adjacent tissue were centrally bisected, paraffin-processed, and embedded on cut surface using routine methods. Five-micron tissue sections were obtained by microtome and stained with hematoxylin and eosin (Poly Scientific Bay Shore, NY) using routine methods.

Results

45 **[0336]** There was minimal ingrowth of tissue into foams (without cells) implanted subcutaneously in SCID mice after 30 days. In contrast there was extensive tissue fill in foams implanted with umbilical-derived cells or placenta-derived cells. Some tissue ingrowth was observed in Vicryl non-woven scaffolds. Non-woven scaffolds seeded with umbilicus- or placenta-derived cells showed increased matrix deposition and mature blood vessels.

50 **[0337] Summary.** Synthetic absorbable non-woven/foam discs (5.0 mm diameter x 1.0 mm thick) or self-assembling peptide hydrogel were seeded with either cells derived from human umbilicus or placenta and implanted subcutaneously bilaterally in the dorsal spine region of SCID mice. The results demonstrated that postpartum-derived cells could dramatically increase good quality tissue formation in biodegradable scaffolds.

EXAMPLE 17**Use of Umbilical Cord Tissue- and Placenta-Derived Cells in Nerve Repair**

5 [0338] Retinal ganglion cell (RGC) lesions have been extensively used as models for various repair strategies in the adult mammalian CNS. It has been demonstrated that retrobulbar section of adult rodent RGC axons results in abortive sprouting (Zeng BY et al. J. Anat. 186:495-508 (1995)) and progressive death of the parent cell population (Villegas-Perez et al., J Neurosci. 8:265-80 (1988)). Numerous studies have demonstrated the stimulatory effects of various exogenous and endogenous factors on the survival of axotomized RGC's and regeneration of their axons (Yip and So, Prog Retin Eye Res. 19: 559-75 (2000); Fischer D et al. Exp Neurol. 172: 257-72 (2001)). Furthermore, other studies have demonstrated that cell transplants can be used to promote regeneration of severed nerve axons (Li et al., 2003; Ramon-Cueto A. et al., Neuron 25: 425-35 (2000)). Thus, these and other studies have demonstrated that cell based therapy can be utilized for the treatment of neural disorders that affect the spinal cord, peripheral nerves, pudendal nerves, optic nerves or other diseases/trauma due to injury in which nervous damage can occur.

10 [0339] Self-assembling peptides (PuraMatrix™, US Patents 5,670,483, 5,955,343, US/PCT applications US2002/0160471, WO 02/062969) have been developed to act as a scaffold for cell-attachment to encapsulate cells in 3-D, plate cells in 2-D coatings, or as microcarriers in suspension cultures. Three-dimensional cell culture has required either animal-derived materials (mouse sarcoma extract), with their inherent reproducibility and cell signaling issues, or much larger synthetic scaffolds, which fail to approximate the physical nanometer-scale and chemical attributes of native

15 ECM. RAD 16 (NH₂-(RADA)₃-COOH) and KLD (NH₂-(KLKD)₃-COOH) are synthesized in small (RAD16 is 5 nanometers) oligopeptide fragments that self-assemble into nanofibers on a scale similar to the in vivo extracellular matrix (ECM) (3D Matrix, Inc Cambridge, MA). The self-assembly is initiated by mono- or di-valent cations found in culture media or the physiological environment. In the protocols described in this example, RAD 16 was used as a microcarrier for the implantation of umbilical cord tissue- and placenta-derived cells into the ocular defect. In this example, it is demonstrated that transplants of umbilical cord tissue- and placenta-derived cells can provide efficacy in an adult rat optic nerve axonal regeneration model.

Methods & Materials

30 [0340] **Cells.** Cultures of human adult umbilicus and placenta derived cells and fibroblast cells (passage 10) were expanded for 1 passage. All cells were initially seeded at 5,000 cells/cm² on gelatin-coated T75 flasks in Growth Medium with 100 Units per milliliter penicillin, 100 micrograms per milliliter streptomycin, 0.25 micrograms per milliliter amphotericin B (Invitrogen, Carlsbad, CA). At passage 11 cells were trypsinized and viability was determined using trypan blue staining. Briefly, 50 microliters of cell suspension was combined with 50 microliters of 0.04% w/v trypan blue (Sigma, St. Louis 35 MO) and the viable cell number, was estimated using a hemocytometer. Cells were then washed three times in supplement free- Leibovitz's L-15 medium (Invitrogen, Carlsbad, CA). Cells were then suspended at a concentration of 200,000 cells in 25 microliters of RAD-16 (3DM Inc., Cambridge, MA) which was buffered and made isotonic as per manufacturer's recommendations. One hundred microliters of supplement free Leibovitz's L-15 medium was added above the cell/matrix suspension to keep it wet till use. These cell/matrix cultures were maintained under standard atmospheric conditions 40 until transplantation occurred. At the point of transplantation the excess medium was removed.

45 [0341] **Animals and Surgery.** Long Evans female rats (220-240 gram body weight) were used. Under intraperitoneal tribromoethanol anesthesia (20 milligram/100 grams body weight), the optic nerve was exposed, and the optic sheath was incised intraorbitally at approximately 2 millimeters from the optic disc, the nerve was lifted from the sheath to allow complete transsection with fine scissors (Li Y et al., 2003, J. of Neuro. 23(21):7783-7788). The completeness of transsection was confirmed by visually observing complete separation of the proximal and distal stumps. The control group consisted of lesioned rats without transplants. In transplant rats cultured postpartum cells seeded in RAD-16 were inserted between the proximal and distal stumps using a pair of microforceps. Approximately 75,000 cells in RAD-16 were implanted into the severed optic nerve. Cell/matrix was smeared into the severed cut using a pair of fine microforceps. The severed optic nerve sheath was closed with 10/0 black monofilament nylon (Ethicon, Inc., Edinburgh, UK). Thus, 50 the gap was closed by drawing the cut proximal and distal ends of the nerve in proximity with each other.

55 [0342] After cell injections were performed, animals were injected with dexamethasone (2 milligrams/kilogram) for 10 days post transplantation. For the duration of the study, animals were maintained on oral cyclosporine A (210 milligrams/liter of drinking water; resulting blood concentration: 250-300 micrograms/liter) (Bedford Labs, Bedford, Ohio) from 2 days pre-transplantation until end of the study. Food and water were available *ad libitum*. Animals were sacrificed at either 30 or 60 days posttransplantation.

[0343] **CTB Application.** Three days before animals were sacrificed, under anesthesia, a glass micropipette with a 30-50 millimeter tip was inserted tangentially through the sclera behind the lens, and two 4-5 microliter aliquots of a 1% retrograde tracer-cholera toxin B (CTB) aqueous solution (List Biologic, Campbell, CA) was injected into the vitreous.

Animals were perfused with fixative and optic nerves were collected in the same fixative for 1 hour. The optic nerves were transferred into sucrose overnight. Twenty micrometer cryostat sections were incubated in 0.1 molar glycine for 30 minutes and blocked in a PBS solution containing 2.5% bovine serum albumin (BSA) (Boeringer Mannheim, Mannheim, Germany) and 0.5% triton X-100 (Sigma, St. Louis, MO), followed by a solution containing goat anti-CTB antibody (List Biologic, Campbell, CA) diluted 1:4000 in a PBS containing 2% normal rabbit serum (NRS) (Invitrogen, Carlsbad, CA), 2.5% BSA, and 2% Triton X-100 (Sigma, St. Louis, MO) in PBS, and incubated in biotinylated rabbit anti-goat IgG antibody (Vector Laboratories, Burlingame, CA) diluted 1:200 in 2% Triton-X100 in PBS for 2 hours at room temperature. This was followed by staining in 1:200 streptavidin-green (Alexa Flour 438; Molecular Probes, Eugene, OR) in PBS for 2 hours at room temperature. Stained sections were then washed in PBS and counterstained with propidium iodide for confocal microscopy.

[0344] Histology Preparation. Briefly, 5 days after CTB injection, rats were perfused with 4% paraformaldehyde. Rats were given 4 cubic centimeters of urethane and were then perfused with PBS (0.1 molar) then with 4% Para formaldehyde. The spinal cord was cut and the bone removed from the head to expose the colliculus. The colliculus was then removed and placed in 4% paraformaldehyde. The eye was removed by cutting around the outside of the eye and going as far back as possible. Care was given not to cut the optic nerve that lies on the underside of the eye. The eye was removed and the muscles were cut exposing the optic nerve this was then placed in 4% paraformaldehyde.

Results

[0345] Lesions alone. One month after retrotubular section of the optic nerve, a number of CTB-labeled axons were identified in the nerve segment attached to the retina. In the 200 micrometers nearest the cut, axons were seen to emit a number of collaterals at right angles to the main axis and terminate as a neuromatous tangle at the cut surface. In this cut between the proximal and distal stumps, the gap was observed to be progressively bridged by a 2-3 millimeter segment of vascularized connective tissue; however, no axons were seen to advance into this bridged area. Thus, in animals that received lesion alone no axonal growth was observed to reach the distal stump.

[0346] RAD-16 transplantation. Following transplantation of RAD-16 into the cut, visible ingrowth of vascularized connective tissue was observed. However, no axonal in growth was observed between the proximal and distal stumps. The results demonstrate that application of RAD-16 alone is not sufficient for inducing axonal regeneration in this situation.

[0347] Transplantation of umbilical cord tissue or placenta-derived cells. Transplantation of cells into the severed optic nerve stimulated optic nerve regrowth. Some regrowth was also observed in conditions in which fibroblast cells were implanted, although this was minimal as compared with the regrowth observed with the transplanted placenta-derived cells. Optic nerve regrowth was observed in 4/5 animals transplanted with placenta-derived cells, 3/6 animals transplanted with adult dermal fibroblasts and in 1/4 animals transplanted with umbilicus-derived cells. In situations where regrowth was observed, CTB labeling confirmed regeneration of retinal ganglion cell axons, which were demonstrated to penetrate through the transplant area. GFAP labeling was also performed to determine the level of glial scarring. The GFAP expression was intensified at the proximal stump with some immunostaining being observed through the reinervated graft.

[0348] Summary. These results demonstrate that transplanted human adult placenta and umbilical cord tissue-derived cells are able to stimulate and guide regeneration of cut retinal ganglion cell axons.

EXAMPLE 18

Use of Umbilicus and Placenta-Derived Cells in Dopaminergic Nerve Repair

[0349] Postpartum umbilicus- and placenta-derived cells were tested for their ability to impart functional improvements in 6-hydroxydopamine (6-OHDA)-lesioned rodents as a model for treating neurodegenerative disease, such as Parkinson's disease (Eisenhofer, G. et al. (2003) FASEB J. 17L1248-1255; Rios M. et al. (1999) J. Neurosci. 1999:3519-26; Xu Y. et al. (1998) J. Neurosci. Res. 54:691-7).

Methods & Materials

[0350] Animal Model and Grouping. Intraparenchymal neurochemical lesioning of the striatum, SNC, or nigrostriatal pathway by 6-hydroxydopamine (6-OHDA) is commonly used as a reliable rodent model for Parkinson's disease. 6-OHDA destroys the dopaminergic neurons, leading to the development of Parkinson's. Two-month old female Sprague-Dawley rats (275-300g) that were to be lesioned with 6-OHDA into the medial forebrain bundle, inducing a parkinsonian phenotype, were purchased directly from Charles River Laboratories (Montreal, Canada).

[0351] Upon arrival, animals were given a period of one week for habituation before undergoing transplantation, and were allowed to feed *ad libitum* throughout the experimental period except during the fasting required by the skilled paw

reaching test described below. Rats were housed two per cage, monitored daily for weight variation and tested during the light phase of a 12:12h light:dark cycle. Animal care and experiments were conducted in accordance with the Canadian Guide for the Care and Use of Laboratory animals and all procedures were approved by the Institutional Animal Care Committee of Laval University. Behavioral deficits associated with the 6-OHDA lesion were evaluated two and half weeks post-surgery by the apomorphine challenge.

[0352] Rotational scores were used to assign animals to four groups. Grafting was performed blind by two investigators involved in this study. Three groups were grafted with different cell types (n=18 per cell type; unknown to the research group), and one group received vehicle (cell culture media) and served as control (n=6). Rats were sacrificed at 4, 8 and 16 weeks following transplantation (n=6 per cell types and n=2 control at each time point). Before each sacrifice, rats were periodically evaluated using three behavioral measures: apomorphine challenge, skilled paw reaching test and head turning.

[0353] Cell Transplants. Cultures of human adult umbilicus-derived, placenta-derived and fibroblast (original) cells (passage 10) were expanded for one passage. All cells were initially seeded at 5,000 cells/cm² on gelatin-coated T75 flasks in Growth Medium. For subsequent passages, all cells were treated as follows. After trypsinization, viable cells were counted after Trypan Blue staining for viability. Briefly, 50 microliters of cell suspension was combined with 50 microliters of Trypan Blue (Sigma, St. Louis MO) and the viable cell number, was estimated using a haemocytometer. Cells were trypsinized and washed three times in DMEM:Low glucose medium (Invitrogen, Carlsbad, CA) (this medium is serum and supplement-free). Cultures of human umbilicus-derived, placenta-derived and fibroblast cells (passage 11) were trypsinized and washed twice in Leibovitz, L-15 medium (Invitrogen, Carlsbad, CA). Cells (2 X 10⁵ cells per injection) were resuspended in 2 microliters of Leibovitz, L-15 medium (Invitrogen, Carlsbad, CA).

[0354] Animal Surgery. All procedures were conducted according to IACUC-approved protocols (Centre de Recherche du CHUL, Local RC-9800, 2705 Blvd Laurier, Ste-Foy, Quebec, Canada G1V 4G2). Transplantation was carried out under ketamine/xylazine (75/10mg/kg i.p.) anesthesia with the animals mounted in a small animal stereotaxic frame (Model 900, David Kopf Instruments, Tujunga, CA). Each transplantation was performed by infusing the cells through a 26-gauge stainless steel beveled needle (45°) attached to a 5 μ l microsyringe (Hamilton Company, Reno, NV) mounted in a motorized microinjection unit (Model UMPII, David Kopf Instruments, Tujunga, CA) infusion pump. Cells (or culture media) were infused into the striatum at a rate of 1.0 μ l/min/site at a average concentration of 100,000 cells/ μ l for a total of 2 μ l per animal according to the following coordinates (if the concentration was lower, volume of injection was adjusted to consistently transplant the same number of cells in all animals): A= 0.5 mm anterior to bregma, L= 3.0 mm lateral to the midline, V= -4.7 mm (site 1) and -4.5 mm (site 2) vertical below dura, with the incisor bar set -2.5 mm below the interaural line. After completion of cell injection, the needle was left in place for an additional 3 min to allow diffusion of the cells before retracting the needle. Rats were treated with 30mg/kg Cyclosporine A (CsA 25mg/mL dilute in olive oil, Bedford Laboratories, Bedford, OH) one day prior to transplantation and given 15mg/kg/day of CsA for the remaining period of the experiment by subcutaneous (s.c.) injection. Animals that served as controls did not receive CsA. All animals received 10 ml of lactate and 0.03 mg/kg of buprenorphine before the surgery as pre-operative treatment and twice a day (lactate once a day) for three days following surgery.

[0355] Apomorphine Rotational Behavior Test. Rats were initially challenged two and half weeks before transplantation and subsequently 2 days prior to the various time points of sacrifice (4, 8 and 16 weeks post-transplantation). Each rat received a dose of 0.05 mg/kg by i.p. injection and was immediately placed in the apomorphine challenge apparatus (spherical bowl). A harness consisting of one elastic placed around the rat's chest just behind the elbows is attached by a Velcro fitting to a rope approximativly 16 inches fitted to a rotometer connected to a computer recording the total number of full body turns made by the rat and the direction of rotation (Rotometer System, San Diego Instruments, San Diego, CA). The final score used for analysis was derived from subtracting the total number of ipsilateral turns from the total number of contralateral turns. Statistical analysis was performed by repeated measures ANOVA using (SAS statistics program).

[0356] Head turning. Animals were tested before transplantation to establish baseline, and then challenged every two weeks following transplantation. Each rat was tested in regard to head position relative to the body for 60 seconds every five minutes three times per challenge. The total number of head deviation of its head (a deviation greater than 10° was considered to be a head turn) was recorded for left and right sides separately and the average number of turn per minute over three minutes was calculated for both sides. Evaluation was made regardless of the rat's activity, including rearing and grooming. Difference between the average number of left and right turns was calculated to determine the behavioral recovery and repeated measures ANOVA were performed as for apomorphine challenge.

[0357] Skilled paw reaching. Skilled forelimb paw reaching ability was assessed at 4, 8 and 16 weeks post-transplantation via a previously published protocol (Moore et al., 2001 *Exp Neurol.* 2001 172(2):363-76). The apparatus is made up of a plexiform glass container that has two compartments. The main chamber (300 mm long X 115 mm high X 103 mm wide), into which the rat is placed, has another sliding component with air holes. A narrower section leads off from this chamber (185 mm X 115 mm X 60 mm) and contains a central 22 mm wide platform that runs along its length, at a height of 62 mm. On both sides of the platform is a 19 mm trough, in which a seven-step staircase is located.

The rat climbs onto the platform and collects 45mg food pellets from small wells within each step. The platform onto which the rat climbs is narrow enough to prevent rat from turning around and reaching to the right trough with the left paw or vice versa. To ensure that the rat does not simply scrape food pellets up the side of the platform, the top of the platform overhangs 5 mm on either side (Moore *et al.*, 2001). This version of the test takes place over 12 days divided into four components: accommodation, training, food deprivation and testing. Accommodation: (days 1-3) rats are put into empty boxes for 20 minutes every day, after which they are taken out of boxes and returned to their home cages. Training: (days 4 & 5) rats are placed in test cages, with staircase 2-6 baited with 6x45 mg food pellets per step for a total of 30 pellets per side for each test session. Rats are left in the apparatus for 20 minutes, after which rats are returned to home cage. Food deprivation: (days 6 & 7) Rats are food deprived and allowed to eat for four hours directly after testing/training every day (water remains all the time). Testing: (days 8-12) each rat is tested for 20 minutes, for five days. Stairs 2-6 are baited, as in the training protocol. After the test, rats are removed from their cages, returned to their home cage and allowed to feed freely for four hours. The number of pellets taken and eaten by each rat is calculated and recorded for both left and right paws separately. The ratios were averaged for the last 5 days of testing, yielding an average accuracy score for each rat and analyze using repeated measures ANOVA.

[0358] Histology. At the time of sacrifice, animals were deeply anesthetized by an i.p. injection of pentobarbital (60 mg/ml, [0.1 ml/100 g]) and perfused intracardially with saline 0.9% containing 0.1% of heparin followed by paraformaldehyde (PFA) 4% in 0.1 M phosphate-buffered saline (PBS) pH 7.4. After perfusion, brains were collected and postfixed for six hours in PFA 4% and then placed in sucrose 20% in PBS. Brains were sectioned 35 μ m-thick on a freezing microtome (Leica Microsystems, Montreal, Canada) and serially collected and stored in antifreeze, subsequently retrieved and washed in PBS for each experiment.

[0359] For immunohistology, sections were washed three times in PBS (0.1M pH 7.4) and pre-incubated in a solution containing 0.4% Triton X-100, 5% NGS in PBS for 60 minutes. Sections were then incubated overnight at 4°C in the primary antibodies according to the following combinations: rabbit anti-Iba-1 (Wako Pure Chemicals Industries, Richmond, VA; 1:1000) and mouse anti-ED1 (Serotech, Raleigh, NC; 1:1000), rabbit anti-glial fibrillary acidic protein (GFAP, DakoCytomation, Mississauga, ON; 1:4000) and mouse anti-human mitochondria (Chemicon, Temecula, CA; 1:500) or rabbit anti-GABA (Chemicon, Temecula, CA; 1:200) (also combined with anti-human mitochondria), diluted in PBS with 0.4% Triton X-100. After washes in PBS, sections were incubated in secondary antibodies; Alexa Fluor® 488 goat anti-rabbit highly cross-adsorbed (Molecular Probes, Eugene, OR; 1:200) and Rhodamine Red-X goat anti-mouse highly cross-adsorbed (Jackson Immunoresearch, West Grove, PA; 1:200) in PBS for two and half hours at room temperature (RT). After washes, sections were incubated in PBS containing 0.022% DAPI (Molecular Probes, Eugene, OR), washed and mounted on gelatin-coated slides, coverslipped with home made DABCO mounting media (polyvinyl alcohol, DABCO, Tris-HCl 1.0 M pH 8.0, distilled water, glycerol) and sealed with nail polish. Fluorescence staining was evaluated using a i90 Nikon fluorescence microscope coupled to a Hamamatsu 1394 ORCA-285 monochrome camera and exploited by Simple PCI software version 5.3.0.1102 (Compix Inc Imaging Systems, PA, USA).

[0360] In the case of double immunofluorescence where both primary antibodies were made in the same hosts, sections were washed in PBS 0.1 M and preincubated in PBS 0.1M containing 1% bovine serum albumin (BSA) and 0.4% Triton X-100 (both from Sigma, St. Louis, MO). Followed an hour of incubation at RT with the first primary antibody; mouse anti-Vimentin (Sigma, St Louis, MO; 1:5000), anti-tubulin isoform β III (Chemicon, Temecula, CA) or mouse anti-Neuron-specific nuclear protein (NeuN, Chemicon, Temecula, CA; 1:5000), sections were washed and incubated an hour in a solution containing secondary antibody FITC-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA; 1:400) in PBS 0.1M, 1% BSA and 0.4% Triton X-100. After washes in PBS, sections were incubated for one hour with 5% normal mouse serum (Jackson Immunoresearch, West Grove, PA), and then washed again before to be incubated with an excess of Fab fragments antibody against the host species of primary antibodies (20 μ g/mL, Jackson Immunoresearch, West Grove, PA) for one hour, further rinsed with PBS. Sections were then incubated one hour at RT with the second primary antibody; mouse anti-human mitochondria (Chemicon, Temecula, CA; 1:500) in PBS containing 1% BSA and 0.4% Triton X-100. Following few washes in PBS, sections were finally incubated with Rhodamine goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA; 1:400) in PBS 0.1 M, 1% BSA, 0.4% Triton X-100 for one hour. Sections were then washed and incubated with DAPI for 7 min (Molecular Probes, Eugene, OR), mounted and coverslipped as described above.

[0361] For tyrosine hydroxylase (TH) immuno-staining, sections were washed three times in PBS 0.1M pH 7.4 and placed in 3% peroxide for 30 min at RT. Slices were subsequently washed in PBS 0.1M and then preincubated in a solution containing PBS 0.1M, 0.1% Triton X-100 (Sigma, St. Louis, MO) and 5% Normal Goat Serum (NGS, Wisent Inc., St-Jean-Baptiste de Rouville, QC) for 30 min at RT. Sections were incubated overnight at 4°C with anti-TH (Pel-Freez, Rogers, AR; 1:5000) in PBS, 0.1% Triton X-100 and 5% NGS. After overnight incubation, sections were washed in PBS 0.1 M and incubated for 1h at RT in a PBS solution containing 0.1% Triton X-100, 5% NGS and biotinylated goat anti-rabbit (Vector Laboratories, Burlington, ON; 1:1500). Following three washes in PBS 0.1 M, sections were placed in a solution of avidin-biotin peroxidase complex (ABC Elite kit, Vector Laboratories, Burlington, ON) for 1h at RT. Antibodies were revealed by placing the sections in Tris buffer solution containing 0.05% 3,3'-diaminobenzidine tetrahy-

drochloride (DAB, Sigma, St.Louis, MO) and 0.1% of 30% hydrogen peroxidase at RT. Reaction was stopped by washing in 0.05M Tris buffer and subsequent PBS washes. Slices were mounted on gelatin-coated slides, air-dried overnight, dehydrated in ascending grades of ethanol and coverslipped with DPX mounting media (Electron Microscopy Science, Hatfield, PA).

5

Results

[0362] Weight Monitoring. Animal weight was monitored daily. As shown in Figure 1, rats demonstrated slow and steady weight gain 2 weeks after transplantation. Weight loss recorded at 3-4, 7 and 15 weeks post-transplantation was feasibly due to fasting required for the staircase test. Continuation of slight weight loss after the first apomorphine challenge was probably due to temporary loss of appetite. Two animals of cell type 1, scheduled to be sacrificed at 8 weeks, were interchanged with two rats scheduled to be sacrificed at 4 weeks due to their progressive weight loss (Rats #27, 49). At 8 weeks post-transplantation, rat #60 of cell type 1 had to be sacrificed one day earlier than scheduled for similar reasons. At 10 weeks post-transplantation, an additional n=2 animals of cell type 2 suffered from diarrhea and demonstrated significant weight loss. These animals were given daily lactate injections but were found dead in the cage 24 hours following these additional precautions. Another two rats of group 2 started to show similar health problems and were preventively sacrificed. The remaining n=2 in group 2 were ultimately sacrificed (24 hours following the last perfusion of group 2 animals). Rat #40 of group 3 was also sacrificed during week 13 of the experimental protocol due to similar health issues as observed in group 2. Rat #66 of group 1 was found dead in its cage at 15 weeks. Prior symptoms resembled those observed in unhealthy animals of groups 2&3. In summary, group 2 animals (n=6 remaining) were sacrificed at 10 weeks post-transplantation, animals of groups 1&3 were sacrificed at 16 weeks post-transplantation as scheduled (n=5 for each group). Abbreviations: BB: Behavioral baseline; TP: Transplantation.

[0363] Apomorphine Challenge. All four groups (cells 1, 2, 3, vehicle) were analyzed using repeated measures ANOVA (variable: no. of rotations), which revealed that only the "time" factor was significant ($p=0.0048$) indicating a "time" effect for all groups. Multiple comparisons showed a significant functional recovery (decrease in number. of rotations) of all groups between time point "0" (baseline) and 4 weeks post-transplantation (Figure 2). This decrease was maintained over time after the 4-week time point. The last time point of 16 weeks post-transplantation could not be analyzed using repeated measures ANOVA since group 2 was sacrificed before this time point (Figure 2).

[0364] A group of five animals, independently lesioned with 6-OHDA, but which did not undergo any surgical intervention (transplantation), was added to the repeated measures ANOVA analysis. When this group is added to the repeated measures ANOVA calculations, only the interaction group/time has a strong tendency towards significance ($p=0.0985$). It is possible that a more significant number of animals would have strengthened this tendency to significant results. Considering that this tendency was indicative of functional recovery over time, further analysis revealed that only umbilical cells (cell 1) induced significant beneficial effects over time ($p=0.0056$) and that lesioned animals which did not undergo intracranial surgical interventions showed a trend towards no functional recovery over time (this result is not significant at $p=0.0655$).

[0365] Head Turning. Head turning depicts the total number of ipsilateral rotations performed by animals following cell transplantation. At the time points tested (2, 4, 6, 8, 10, 12, 14, and 16 weeks post-transplant) no significant differences were observed between animals transplanted with cells or animals that received vehicle alone (Figure 3). Head turning is purely subjective and determines the natural tendency of the rat to rotate its head left or right after lesion. All groups were analyzed using repeated measures ANOVA (variable: difference between the number of left and right head turns). As time increased in this study an improvement was seen in terms of no bias for head turning, however this was apparent in all groups (Figure 3).

[0366] Staircase Test. No significant differences between transplanted or control groups were identified using this test. The staircase test measured food intake in 20-minute testing periods, which requires fine movements to pick up food in the stairs. All groups were analyzed using repeated measures ANOVA (variable: ratios of eaten to taken pellets). Using this test it was determined that over time a difference in eating behaviors was observed however, no significant difference could be determined between groups (Figure 4).

[0367] Immunostaining. H&E sections demonstrated good cellular engraftment 1 day post-transplant. Cells were identified in the transplant sites up to 8 weeks post-transplantation by human nuclear antigen staining although the numbers of human cells had decreased in response to time in the graft. At present, no data has confirmed that postpartum-derived cells differentiated into a neuronal phenotype following transplantation *in vivo* in this model system.

[0368] Cell grafts were analyzed for the presence of the microglial marker Iba-1. As shown in Figure 5a, Iba-1 was abundantly expressed by each cell type, especially relative to vehicle controls. Iba-1 expression was found to tend toward decline over time (Figure 5a). Assessment of ED-1 in transplanted animals demonstrated that a macrophage response was evident following transplantation, the level of staining of ED-1 decreased in all groups except animals transplanted with fibroblasts over time (Figure 5b). DAPI staining generally remained constant throughout the duration of the study (Figure 5c).

[0369] Similarly, determination of levels of glial fibrillary acidic protein (GFAP) in the grafts to determine numbers of reactive astrocytes following grafting demonstrated that initially reactive astrocytes were identified following transplant or vehicle administration an effect that diminished over time (Figure 6a). Consistent with other observations showing differentiation of the cells in the graft, Vimentin was found to be abundantly expressed in the cells four weeks following the graft, but expression steadily declined over the next twelve weeks (Figure 6b).

[0370] Staining for human tyrosine hydroxylase was negative. Therefore, neither umbilical nor placental cells differentiated into dopaminergic cells under the treatment conditions used.

Summary

[0371] The results demonstrated that implantation of umbilical cells provided functional improvement over time in a 6-OHDA model of Parkinson's as assessed by behavioral responsiveness to apomorphine challenge. Both the staircase and head turning tests were performed to determine effects on the activation of different neuronal circuitries/and or mechanisms. No differences were identified using these test parameters, thus as yet a mechanism relating to the positive benefit seen at 4 and 8 weeks in animals receiving umbilical transplants remains unresolved.

[0372] Immunohistochemical staining demonstrated no evidence of cell differentiation following cell engraftment. No neuronal, or more specifically no dopaminergic differentiation could be demonstrated in these studies. Thus, no evidence for cell differentiation in the graft site could be verified. This further suggests that the improvements observed in behavior by apomorphine challenge following umbilical cell transplant are likely due to a trophic response and not a result of regenerative cell potential.

[0373] TH-immunopositive cells were not observed in any cell type at any time points. However, TH is not the sole cellular pathway for producing dopamine. Dopamine can be produced independently from tyrosine hydroxylase, via the tyrosinase pathway. Moreover, dopamine, in the presence of tyrosinase, covalently modifies and inactivates tyrosine hydroxylase. In addition, transplanted cells may enable the processing of DOPA (amino acid from food) in plasma after a meal to DOPA.

EXAMPLE 19

RayBio® and BD Powerblot™ Cytokine Arrays

[0374] RayBio® Human Cytokine Antibody Array C Series 1000 was used to analyze the expression of 120 proteins in postpartum-derived cells and lysates. This analysis provided a characterization of the UTCs and identified an expression spectrum of key trophic factors for these cells.

Materials and Methods

[0375] **Cell Growth and Harvest.** Umbilical-derived cells were seeded at 5,000 cells per cm² in gelatin-coated flasks with growth media and expanded for 3 to 4 days (25,000 cells per cm² target harvest density). Cells were harvested with trypsin, collected, and centrifuged at 300 rcf for 5 minutes. The trypsin/media was removed by aspiration and cells were washed three times with phosphate buffered saline (PBS).

[0376] **Cell Wash and Aliquoting.** After washing, the cells were re-suspended at 10⁷ cell/ml in PBS and delivered as 1 ml aliquots into 1.5 ml sterile siliconized microcentrifuge tubes. The cells were centrifuged at 300 rcf for 5 minutes and the PBS was removed by aspiration. Cells were either lysed and analyzed by the array, or lysed and lyophilized for analysis.

[0377] **Preparation of Lyophilized Samples.** Three lots of cells (UTC Lots L040405, L052505, L050505) were prepped for eventual lyophilization by immersing into liquid nitrogen (LN2) for 60 seconds. The tubes were then removed from LN2 and immediately immersed in a 37°C water bath for 60 seconds or until thawed (3 minute maximum incubation time). This process was repeated two additional times. The freeze-thawed samples were centrifuged for 10 minutes at 13,000 rcf at 4° C and placed on ice. The supernatant fluid from each tube was removed. To determine total protein content, lysate was diluted into PBS and the dilution was analyzed by Bradford assay.

[0378] For lyophilization, multiple 1.5 ml sterile cryovials labeled with lysate were loaded into an autoclaved and cooled heat transfer block. Aliquots of lysate supernatant fluid at defined total protein concentration were loaded into the cryovials. The heat block containing uncapped cryovials were aseptically loaded into autoclaved un-used autoclave pouch. The pouch was loaded into the lyophilizer.

[0379] Test materials with applied lysate were loaded into a FTS Systems Dura-Stop MP Stoppering Tray Dryer and lyophilized using the following ramping program. All steps had a ramping rate of 2.5°C/minute and a 100-mT vacuum.

Step	Shelf Temperature (°C)	Hold Time (minutes)
a	-40	180
b	-25	2160
c	-15	180
d	-5	180
e	5	120
f	20	120
g	20	60

15 **[0380] Preparation of Cell Pellets.** Frozen cell pellets (lots 063004B, 022803, 050604B, 072804, 120304, 071404C, 090304) were lysed using a 1:1 mix of RIPA buffer (50 mM Tris HCl, pH8, 150 mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate and 0.1% SDS) and cell lysis buffer provided in the RayBio® cytokine array 1000.1 kit (Raybiotech Inc. Norcross, GA). Glass beads (Sigma, MO) were used to achieve complete cell lysis. Protein concentration was measured using the BCA protein assay kit (Pierce Biotechnology, Inc. Rockford, IL).

20 **[0381] RayBio® Array Analysis.** RayBio® arrays VI and VII, which constitute the array 1000.1, were probed overnight with equal amounts of protein from each sample. The remaining protocol was followed as per the manufacturer's guidelines. The spots on the membrane were qualitatively analyzed to determine proteins of interest. For quantitative comparison between samples, these spots could be analyzed by densitometry and changes in expression confirmed by ELISA.

25 **Results**

30 **[0382]** A total of ten different UTC populations were analyzed. Forty-eight proteins were qualitatively identified and listed in Table 19-1. Some proteins were expressed at relatively high concentrations in all samples tested while others were expressed in certain samples.

Table 19-1. Qualitatively identified UTC proteins.		
Number	Trophic Factor	Abbreviation
1	Brain Derived Neurotrophic Factor	BDNF
2	Basic Fibroblast Growth Factor	bFGF
3	Bone Morphogenetic Protein-4	BMP-4
4	Bone Morphogenetic Protein-6	BMP-6
5	MPIF-1	CK b 8-1
6	Ciliary Neurotrophic Factor	CNTF
7	CCL27 (cutaneous T cell attracting chemokine)	CTACK
8	Epidermal Growth Factor	EGF
9	CCL26	Eotaxin-3
10	Fas Antigen	Fas/TNFRSF6
11	Fibroblast Growth Factor-6	FGF-6
12	FMS related Tyrosine Kinase 3	FIT-3 ligand
13	CS3C chemokine	Fractalkine
14	Granulocyte Colony Stimulating Factor	GCSF
15	Glucocorticoid Induced TNF Receptor Superfamily-Related Protein	GITR ligand
16	Granulocyte-Macrophage Colony Stimulating Factor	GM-CSF
17	Hepatocyte Growth Factor	HGF

(continued)

Table 19-1. Qualitatively identified UTC proteins.

Number	Trophic Factor	Abbreviation
18	CCL1	I-309
19	Intercellular Adhesion Molecules 1	ICAM-1
20	Insulin Like Growth Factor Binding Protein-1	IGFBP-1
21	Insulin Like Growth Factor Binding Protein-2	IGFBP-2
22	Insulin Like Growth Factor Binding Protein-3	IGFBP-3
23	Insulin Like Growth Factor Binding Protein-6	IGFBP-6
24	Interleukin-10	IL-10
25	Interleukin-13	IL-13
26	Interleukin-1a	IL-1a
27	Interleukin-1Ra	IL-1Ra
28	Interleukin-3	IL-3
29	Interleukin-5	IL-5
30	Interleukin-6	IL-6
31	Interleukin-7	IL-7
32	Interleukin-8	IL-8
33	IFN-Inducible T Cell Chemoattractant	I-TAC
34	Monocyte Chemotactic Protein-1	MCP-1
35	Migration Inhibitory Factor	MIF
36	Macrophage Inflammatory Protein-1	MIP-1a
37	Oncostatin M	Oncostatin M
38	Phosphatidylinositol Glycan F	PIGF
39	Soluble gp130-signal transducer chain	sgp130
40	Transforming Growth Factor-B1	TGF-b1
41	Transforming Growth Factor-B3	TGF-b3
42	Thrombopoietin	Thrombopoietin
43	Tissue Inhibitor of Metalloproteinase 2	TIMP-2
44	Tumor Necrosis Factor-alpha	TNF-a
45	Tumor Necrosis Factor-beta	TNF-b
46	TNF-Related Apoptosis-Inducing Ligand Receptor-3	TRAIL-R3
47	TNF-Related Apoptosis-Inducing Ligand Receptor-4	TRAIL-R4
48	Urokinase-Type Plasminogen Activator Receptor	uPAR

Summary

[0383] The RayBio® array confirms the expression of proteins previously identified by gene array and/or ELISA analyses. Various trophic factors beneficial for specific disease treatment have been identified. For instance, FGF, TGF-b, and GCSF were identified in UTCs, and these growth factors have been previously identified with improvements in animal models of acute stroke and stroke recovery. In addition, BDNF, BMP-4, BMP-6, and TGF-b1, which are positively associated with Parkinson's disease, have been identified in UTCs. All data presented are qualitatively assessed; quan-

titative analysis of the level of expression for proteins of interest is pending.

EXAMPLE 20

5 Telomerase Expression in umbilical-derived cells

[0384] Telomerase functions to synthesize telomere repeats that serve to protect the integrity of chromosomes and to prolong the replicative life span of cells (Liu, K, et al., PNAS, 1999; 96:5147-5152). Telomerase consists of two components, telomerase RNA template (hTER) and telomerase reverse transcriptase (hTERT). Regulation of telomerase is determined by transcription of hTERT but not hTER. Real-time polymerase chain reaction (PCR) for hTERT mRNA thus is an accepted method for determining telomerase activity of cells.

[0385] **Cell Isolation.** Real-time PCR experiments were performed to determine telomerase production of human umbilical cord tissue-derived cells. Human umbilical cord tissue-derived cells were prepared in accordance the examples set forth above. Generally, umbilical cords obtained from National Disease Research Interchange (Philadelphia, Pa.) following a normal delivery were washed to remove blood and debris and mechanically dissociated. The tissue was then incubated with digestion enzymes including collagenase, dispase and hyaluronidase in culture medium at 37°C. Human umbilical cord tissue-derived cells were cultured according to the methods set forth in the examples above. Mesenchymal stem cells and normal dermal skin fibroblasts (cc-2509 lot # 9F0844) were obtained from Cambrex, Walkersville, Md. A pluripotent human testicular embryonal carcinoma (teratoma) cell line nTera-2 cells (NTERA-2 cl.DI), (see, Plaia et al., Stem Cells, 2006; 24(3):531-546) was purchased from ATCC (Manassas, Va.) and was cultured according to the methods set forth above.

[0386] **Total RNA Isolation.** RNA was extracted from the cells using RNeasy® kit (Qiagen, Valencia, Ca.). RNA was eluted with 50 microliters DEPC-treated water and stored at -80°C. RNA was reverse transcribed using random hexamers with the TaqMan® reverse transcription reagents (Applied Biosystems, Foster City, Ca.) at 25°C for 10 minutes, 37°C for 60 minutes and 95°C for 10 minutes. Samples were stored at -20°C.

[0387] **Real-time PCR.** PCR was performed on cDNA samples using the Applied Biosystems Assays-On-Demand™ (also known as TaqMan® Gene Expression Assays) according to the manufacturer's specifications (Applied Biosystems). This commercial kit is widely used to assay for telomerase in human cells. Briefly, hTERT (human telomerase gene) (Hs00162669) and human GAPDH (an internal control) were mixed with cDNA and TaqMan® Universal PCR master mix using a 7000 sequence detection system with ABI prism 7000 SDS software (Applied Biosystems). Thermal cycle conditions were initially 50°C for 2 minutes and 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. PCR data was analyzed according to the manufacturer's specifications.

[0388] Human umbilical cord tissue-derived cells (ATCC Accession No. PTA-6067), fibroblasts, and mesenchymal stem cells were assayed for hTERT and 18S RNA. As shown in Table 20-1, hTERT, and hence telomerase, was not detected in human umbilical cord tissue-derived cells.

Table 20-1		
	hTERT	18S RNA
Umbilical cells (022803)	ND	+
Fibroblasts	ND	+
ND- not detected; + signal detected		

[0389] Human umbilical cord tissue-derived cells (isolate 022803, ATCC Accession No. PTA-6067) and nTera-2 cells were assayed and the results showed no expression of the telomerase in two lots of human umbilical cord tissue-derived cells while the teratoma cell line revealed high level of expression (Table 20-2).

Cell type	hTERT		GAPDH		hTERT norm
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	
nTera2	22.85	27.31	16.41	16.31	0.61
022803	-	-	22.97	22.79	-

[0390] Therefore, it can be concluded that the human umbilical tissue-derived cells of the present invention do not express telomerase.

EXAMPLE 21

5

Use of Umbilical Cord Tissue-Derived Cells in Treatment of ALS

[0391] This study evaluated the disease modifying effects of human umbilical cord tissue derived cells ("hUTC") either at the onset or one week following onset of disease to mimic the clinical paradigm in which patients presenting symptoms of ALS might be administered hUTC. In particular, the objective of this study was to determine the efficacy of human umbilical cord tissue derived cells ("hUTC") in an SOD1 G93A rat model of Amyotrophic Lateral Sclerosis (ALS). The effect of administration of hUTC on animal survival, and locomotor function was evaluated after hUTC delivery via intrathecal injection.

[0392] The rationale for local delivery of hUTC via intrathecal injection was to deliver the cell right at the site of disease initiation. In addition, this route of administration bypasses the blood-brain barrier, allowing rapid access to potential reactive sites for the test article in the spinal cord (Ochs G et al. (1999) J Pain Symptom Manage. 18:229Y32). Functional evaluations of locomotor activity were performed once weekly in the Basso, Beattie and Bresnahan (BBB) test (Basso DM et al. (1996) Exp. Neurol. 139: 244-256) and inclined plane performance test (Rivilin AS, Tator CH. (1977) J Neurosurg 47(4): 577; Lindberg RL et al. (1999) J. Clin Invest. 103(8):1127-1134).

[0393] Currently the SOD 1 G93A rodent models are the only type of animal model for ALS. It is a useful, cost-effective method for screening new therapies for the disease as demonstrated by other investigators for efficacy of various agents/compounds on ALS (Karussis et al. (2010) Arch Neurol. 67(10):1187-94; Xu et al. (2009) J Comp Neurol. 514(4):297-309; Xu et al. (2006) Transplantation. 82(7):865-75; Garbuzova-Davis S et al. (2003) J. Hematother Stem Cell Res. 12(3):255-70; Galán et al. (2010) Neurologia. 25(8):467-469; Kim et al. (2010) Neurosci Lett. 468(3):190-4; Gros-Louis et al. (2010) J Neurochem. 113(5):1188-99; Israelson A et al. (2010) Neuron. 26; 67(4):575-87).

Methods & Materials

[0394] SOD1 G93A male rats supplied by Dr. David S. Howland, University of Washington, Seattle, were bred to N (SD) female rats from Taconic (Germantown, NY). Rats were bred for one week in pairs of one male: one female. Offspring were weaned and genotyped at 21 days of age and positive transgenic pups were identified for enrollment into the study. Colony was propagated by back-breeding male pups of the same litter with the original female breeders to reduce phenotypic variance. All animal care and surgery procedures in this study were carried out according to protocols approved by the Animal Care and Use Committee of the Johns Hopkins Medical Institutions.

[0395] hUTC (isolated from human umbilical cord tissue) were thawed from frozen stock as described in U.S. Patent No. 7,510,873 and diluted into phosphate buffered saline (PBS). The cells were centrifuged at 1500 X g for 5 minutes, then resuspended in PBS again and counted. The cell concentration was adjusted to 20 million cells per milliliter and 50 micro liter of this suspension was delivered to each rat intrathecally.

[0396] The animals positive for the superoxide dismutase 1 (SOD1) gene were enrolled into the study. These animals were weighed twice a week to determine when their body weight dropped for two consecutive measurements, the time of which was defined as disease onset. This is a sensitive and very objective measure for determining disease onset in rodent models of ALS (Xu et al. (2006) Transplantation. 82(7):865-75). Rats with disease onset were randomly assigned in pair wise to vehicle or cell treated groups.

[0397] The study had two arms (Table 21-1). Each arm contained two groups. Arm 1 Group 1 received a single intrathecal injection of Phosphate Buffered Saline (PBS, vehicle control) at time of disease onset. Arm 1 Group 2 was treated with 1 million hUTC cells at approximate disease onset. Cells were delivered in PBS via intrathecal injection. Arm 2 Group 3 was treated with a single intrathecal injection with PBS (vehicle control) at 1 week after approximate disease onset. Arm 2 Group 4 was treated with recombinant with 1 million hUTC cells at 1 week after approximate disease onset. Cells were delivered in PBS via intrathecal injection.

50

Table 21-1. Treatment Groups

	Treatment group	Number of animals	Gender	Test material/Time of Injection	No. of cells injected
Arm 1	1	10	Mixed	PBS (at disease onset)	0
	2	10	Mixed	hUTC (at disease onset)	1.0 x 10 ⁶

(continued)

Table 21-1. Treatment Groups

	Treatment group	Number of animals	Gender	Test material/Time of Injection	No. of cells injected
5 Arm 2	3	10	Mixed	PBS (at 1wk after disease onset)	0
	4	10	Mixed	hUTC (at 1wk after disease onset)	1.0 x 10 ⁶

*PBS was used as vehicle in this study

10 [0398] To minimize injury and maximize prompt recovery of the animal, a transcutaneous injection method was employed to deliver cells intrathecally into the CSF of the lumbar cistern of SOD1 rats. Animals were mounted on Kopf stereotaxic device and anesthetized with gas anesthesia (isoflurane: oxygen: nitrous oxide = 1:33:66). The lumbar region was shaved and a cylindrical support element placed under the rat lower abdomen to stretch the intervertebral spaces of the lumbar spine. Cells were delivered with 200-500 μ l microsyringes fitted with a 25Gx1" needle. The needle with microsyringe containing 50 micro liter cells was slowly inserted into the subarachnoid space at the L4-5 or L6-S1 intervertebral spaces. When a physical sign of irritation of the cauda equina was observed (i.e. tail or hind paw flick), cells were slowly infused into the subarachnoid space over a 1-minute period. Syringe was held in place for 2 minutes and then the needle was withdrawn. The needle entry point was pressed with a cotton swab tip immersed in iodine for 1 minute, and then the animal was dismounted and put on a heat pad to allow recovery. After the animal woke up, it was returned to its cage.

15 [0399] The disease progression and potential therapeutic efficacy were monitored through two functional evaluations: (1) locomotor activity (measured weekly); and (2) body weight (measured twice a week). The locomotor tests included the Basso, Beattie and Bresnahan (BBB) test (Basso et al. (1995) J Neurotrauma 1995; 12(1): 1; Basso et al. (1996) Exp. Neurol. 139: 244-256) and the inclined plane performance test (Rivlin AS, Tator CH. (1977) J Neurosurg 47(4): 577).

20 [0400] For BBB scoring, the rats were gentled and adapted to the open field. Once a rat walked continuously in the open field, two examiners, blinded for the treatment, conducted a 4-minute testing session using the BBB locomotor rating scale that is a 21-point scale with high intra- and interobserver reproducibility. The open field test was videotaped. From this tape, two examiners quantitatively assessed the number of hindlimb (HL) movements performed during a 1-minute period; this time period was a composite of episodes over the 4-minute testing session when each HL is in full view of the camera. The 1-minute time limit ensured that the right and left HLs of each animal were assessed for the same length of time since both HLs are not always in full view of the camera. The footage was reassessed in slow motion if the examiners' scores disagreed. Absence of observable hind limb movement was scored 0 and the initial points were awarded for isolated joint movements. The score increased when more joints showed movement and/or the movements were more extensive. As locomotion increased, points were given for plantar placement of the paw, weight support and coordination between forelimb and hind limb. The final points were achieved by toe clearance, trunk stability and tail position.

25 [0401] The inclined plane is another behavioral task that assesses the animal's ability to maintain its position on a rubber corrugated board; this board was raised at 5° increments. The maximum angle at which an animal can support its weight for 5 seconds is defined as the capacity angle. This test examines sensory feedback, coordination, and muscle strength required for locomotion.

30 [0402] The end-stage of the disease was determined by paralysis so severe that the animal could not right itself within 20 seconds when placed on its side. Both BBB and inclined plane scores at this point are zero, which is an endpoint frequently used for SOD1 mutant rats and one that is consistent with the requirements of the Animal Care and Use Committee of Johns Hopkins University.

35 [0403] BBB and inclined plane scores were analyzed by repeated measures analysis of variance (ANOVA) followed by Fisher LSD post-hoc test to assess differences between vehicle and cell treated groups. Course of illness as an effect of treatment was analyzed by comparing age at disease onset and age of death between the two groups (with student's t test) as well as with Kaplan-Meier survival analysis followed by log-rank testing.

40 **Histological analysis**

45 [0404] Host motor neuron status was explored by cresyl violet staining of motor neurons in the lumbar protuberance (L4-5) (data not shown). Motor neuron number is greatly decreased and many neurons appear as degenerating profiles in both stem cell and control groups, a pattern consistent with the fact that all spinal cord tissues are acquired at end-disease stage. No surviving stem cells were found inside the spinal cord parenchyma using ICC staining with human specific anti-nuclear antibody. L4-L5 ventral roots from both groups were studied by Toluidine blue staining. Axon degeneration and demyelination were significant and similar in both stem-cell and control groups, consistent with the

acquisition of these preparations at the terminal stage of disease.

Results

5 [0405] The Kaplan-Meier plots show an increase in animal survival for the the cell treated group compared to the control animals (n=10 for groups 1&2, n=8 for groups 3&4) throughout the course of treatment, initiated at either disease onset (Figure 7A, Arm 1) or one week after onset (Figure 7B, Arm 2), except one animal in Group 1 that lived longer than the rest of animals in the study (Figure 7A). While the average time of disease onset was not different between animals that received vehicles and animals that were injected with hUTC in both arms (Figure 8), the average life span increased following intrathecal administration of hUTC, especially in Arm 2 in which animals had a longer life span by 15.75 days (2.25 weeks, P<0.035) than the vehicle group although the age of disease onset is similar.

10 [0406] Figure 9 shows that administration of hUTC at disease onset (Figures 9A and 9B) or 1 week following disease onset (Figures 9C and 9D) was associated with increased scores in the BBB test and inclined plane performance test, indicating a slower progression in muscle weakness in animals grafted with hUTC compared to animals that received vehicle only. These results suggest that hUTC might be able to preserve locomotor activity in this model.

15 [0407] Thus, the data disclosed in this example, summarized in Table 21-2 below, show that intrathecal injection of hUTC can preserve motor neuron function and extend life in the rat model of ALS.

Table 21-2: Time of disease onset and duration				
	Treatment group	Disease onset Days (Weeks)	Disease duration Days (Weeks)	Life span Days (Weeks)
20 Arm 1	1. PBS	130.5 + 6.9 (18.64 + 0.99)	30.9 + 17 (4.41 + 2.43)	161.4 + 14.4 (23.06 + 2.06)
	2. hUTC	129.4 + 9.0 (18.49 + 1.29)	39.2+10.4 (5.6 + 1.49)	168.6 + 15.4 (24.09 + 2.20)
25 Arm 2	3. PBS	122.0 + 8.4 (17.43 + 1.2)	26.9 + 6.8 (3.84 + 0.97)	148.9 + 10.7 (21.27 + 1.53)
	4. hUTC	123.8 + 8.7 (17.69 + 1.24)	40.9 + 19.3 (5.84 + 2.76)	164.6 + 15.8* (23.51 + 2.26)
30 Notes: * P<0.035				

Definitions for Table 21-2

[0408]

40 **Disease onset:** The point at which body weight will be found decreased for two consecutive times. This is a sensitive and very objective measure for determining disease onset in rodent models of ALS (Xu L. et al. (2006)).

Determination of End-stage of the disease: The stage at which paralysis is so severe that the animal could not right itself within 20 s when placed on its side (an endpoint frequently used for SOD1 mutant rats) and animals have to be euthanized (Israelson et al, 2010).

Disease Duration: The period from the time of disease onset and the end stage of the disease

Life span: The period between the time of birth and euthanization.

EXAMPLE 22

Use of Umbilical Cord Tissue-Derived Cells in the Preventative Treatment of ALS

50 [0409] The disease modifying effects of hUTC were evaluated prior to disease onset (10 or 12 weeks of age) as a preventative treatment. The cells were administered via an intravenous tail vein injection, which represents a clinically advantageous route of administration. Functional evaluations of locomotor activity were performed once weekly in the Basso, Beattie and Bresnahan (BBB) test and inclined plane performance test.

Purpose and Scope of the Study

[0410] The goal of this study was to explore the preventative role of umbilical cord-derived, self-renewable and expandable cells, on clinical aspects of transgenic rat ALS as it occurs in SOD1 G93A rat subjects, along the main methodological lines published in work by Koliatsos and colleagues (Yan et al. (2006) *Stem Cells* 24: 1976-1985; Yan et al. (2007) *PLoS Med* 4: e39; Xu et al. (2006) *Transplantation* 82:865-875). Two arms were chosen: (1) a 10-week arm that received the umbilical cord-derived cells or vehicle at 10 weeks of age; and (2) a 12-week arm that received cells or vehicle at 12 weeks of age. Within each arm, Group A (blinded in most cases as odd-numbered animals) was designated to receive the umbilical cord-derived cells and Group B (blinded in most cases as even-numbered animals) was delegated to vehicle treatment. No immune protection of graft-versus-host events as proposed in earlier studies by the Koliatsos Lab (Yan et al. (2006); Xu et al. (2006)) was performed. The analysis that follows does take into account the shifting phenotype of the colony from a very variable to a less variable and eventually optimized population with respect to disease onset and severity.

15 Breeding and phenotype optimization-observations on disease onset and life span

[0411] SOD1 rat colony was propagated by back-breeding male pups of the same litter with the original female breeders to reduce phenotypic variance. The standard was that the male breeder's disease onset time should be within 90-120 days. Otherwise, the phenotype of its progenies was observed to become "unstable", i.e. disease onset could drift as far as 180 days of age and beyond. The initial breeders that had been used to generate animals used for this study were outside this 90 to 120 days frame, a problem that caused substantial phenotypic drift in their progeny. To decrease the impact of phenotypic drift, each animal designated to group A was initially paired with an animal of the same gender from the same litter designated to group B. Average disease onset times for groups A and B in the 10-week arm were 153.15 ± 12.71 and 154.0 ± 13.75 days and life spans for groups A and B were 202.0 ± 10.32 , 202.75 ± 17.31 days, respectively. The average disease onset time for groups A and B in 12-week arm were 149.0 ± 27.58 and 146.5 ± 28.53 days; life spans for groups A and B were 180.5 ± 30.25 , 174.88 ± 35.87 days, respectively. Although both average disease onset time and life span in group A were longer than in group B, these differences were not statistically significant. Because the last two pairs in the 12-week arm were comprised of genotypically tight SOD1 rats, i.e. progenies of SOD1 rats with disease onset times within the 90-120 day frame, these rats were analyzed separately from other rats in the same arm. The average disease onset time of these two pairs in group A and B were 114.5 ± 0.5 and 111.0 ± 0.61 days, a difference that was not statistically significant. Life spans of subjects from these two pairs delegated to group A were 133.5 ± 0.03 , i.e. 10 days longer than life spans of subjects in group B (123.0 ± 0.41 days).

35 Survival Curve Patterns

[0412] Kaplan-Meyer curves studied with log-rank analysis did not show significant differences between groups A and B in the 10- and 12-week arm experiments (Figure 10 and Figure 11). However, in the 12-week arm experiment, there was a tendency of group A to show improved survival; median survival times for group A and B were 194 and 185 days, respectively (see Figure 11). Although there was no significant difference between the last two pairs in the 12-week arm experiment, survival curve analysis shows the promising trend that group A may survive longer (Figure 12).

[0413] **Behavioral testing of animals in the two arms--inclined plane and BBB data:** Serial data from these two tests with repeated-measures ANOVA (RM ANOVA) was analyzed in two ways. In the first mode, data starting at one week before the first animal showed signs of disease was collected and comparison analysis was performed (left top panels in Figures 13-18). In the second mode, data was collected starting at the time point of disease onset (left bottom panels in Figures 13-18). When performing these analyses, a "compensatory" correction was also applied to remove the curve deviation factor caused by the very disparate survival curves (because of genotypic variance) (right panels in Figures 13-18). In applying this compensation, the lowest behavior test score from the animal near the time of its death was taken and entered along with the scores of surviving animals from the same group in subsequent time points (an alternative method would be to remove this data point from later behavioral data sets and do the analysis on a smaller n of animals). RM ANOVA results on inclined plane and BBB data did not show significant differences between the two groups in the 10- and 12-week arm experiments, corrections and adjustments for disease onset and survival notwithstanding (Figures 13-15, 17). However, when the last two pairs from the 12-week arm experiment were analyzed for reasons explained in the breeding section above, the behavioral results were found to have similar variance to the ones from the survival study (see above; Figures 16, 18). Although there was no statistical difference on the behavioral scores between these two pairs, there was a trend for better performance of group A over group B.

Histological analysis

[0414] All SOD1 rats with or without stem cell injections were killed when their disease was at end stage (BBB score <3). After perfusion with 4% PFA, the animal's brain and other organs were collected, cryoprotected and frozen for further processing. To explore whether there were any remaining human stem cells on sections from relevant organs/tissues, immunocytochemistry with the human-specific nuclear (HNU) antibody was performed. These tissues included lumbar spinal cord (as a relevant therapeutic target) and lung (as organ control with anticipated high concentration of injected stem cells). HNU staining did not show any survival for grafted cells in these organs in end-stage animals.

[0415] **Observations on spinal cord sections:** G93A SOD1 rats recapitulate many cardinal symptoms of human ALS. Motor neuron degeneration in SOD1 rat spinal cord proceeds from lumbar motor neurons that innervate lower limb muscles to cervical and thoracic motor neurons that innervate respiratory muscles. Animals die from inanition and respiratory failure. The histological aim of this experiment was limited because of the lack of the appropriate tissues from mid-disease animals and a substantial phenotypic spread. Available spinal cord tissues were from animals killed at end stage, with the majority of motor neurons having degenerated, even in animals with hypothetical protective effects of stem cells injection. Therefore, the only value of histological analysis was to depict gross neuropathology in the spinal cords of animals treated with live or dead cells. Five such spinal cords were processed. Six pairs of sections, equally spaced every 300 μ m across the L4-6 region of lumbar cord, were studied from each. One section from the pair was stained with cresyl violet and the other with hematoxylin and eosin. Three cases were taken from odd-number and two cases from even-number animals.

[0416] Stained sections reveal normal gross spinal cord appearance with all major segments of spinal cord clearly delineated (Figure 19A and Figure 19B). No tumors were detected (Figure 19A and Figure 19B). As predicted based on the motor neuron phenotype of SOD1 G93A animals, there was profound loss of a motor neurons in both even- and odd-number animals. When the numbers from the two groups based on numbers of nucleoli present in basophilic profiles of the ventral horn at 100X magnification were counted, there were no appreciable trends in motor neuron cell numbers between the two groups (odd-number animals, 423; even-number animals, 418). Abercrombie's adjustment for split-cell error was used to correct for possible differences in nucleolar diameter between the two groups ($N_i = n_i \cdot t / t + d$, where n_i = number counted, t = section thickness, and d = average profile diameter).

[0417] Evaluation of BBB and scores from cell treated animals show trending better than untreated animals. The results of this study therefore suggest times of administration closer to or after disease on-set may have better therapeutic efficacy.

EXAMPLE 23**Trophic Factors for Neural Progenitor Differentiation**

[0418] Neural stem cells in the central nervous system (CNS) can self renew and are multipotent as they can differentiate into neurons, oligodendrocytes and astrocytes. These cells may be activated following an insult and initiate neurogenesis. However, this differentiation efficiency is very low and the newly generated neurons are mostly short-lived, possibly due to the absence of the formation of any functional synapses. Exogenous delivery of cell-based products may enhance neurogenesis and the formation of new synapses and consequently overcome this issue and thus may improve functional recovery in trauma or degenerative conditions. Recent studies have suggested that cell based technologies may be an effective therapy for multiple neurological conditions. To understand the underlying mechanism(s), the effect of hUTC on adult neural stem differentiation was determined.

Materials & Methods**Cell culture.**

[0419] Human umbilical tissue-derived cells (hUTC) were thawed (lot no. 22042008, at passage P3 or P4) and seeded onto T75 and/or T225 flasks and cultured in Hayflick growth medium (DMEM - low glucose (Gibco, Catalog number 11885-084), 15% v/v fetal bovine serum (FBS, Hyclone catalog number SH30070.03IR), 4 mM Glutamax (Gibco, catalog number 3505-061) and 1% penicillin/streptomycin (Gibco catalog number 15070063) overnight or for 48 hours to form a confluent layer. After the cells reached confluent layer, they were washed three times with phosphate buffered saline (PBS, Invitrogen, catalog number 25200-056) and incubated with DMEM serum free medium for 8 hours or 24 hours as indicated. Cells were detached using TrypLE™ (Gibco, Catalog number 12604-021) and quantitated using Guava® instrument (Guava Technologies).

[0420] Adult rat hippocampal neural stem cells (Lot no. R0706F0009, Millipore, catalog number SCR022) were thawed (P3 or P4), seeded onto poly-L-ornithine (Sigma, catalog no. P3655) and laminin (Sigma, catalog number 2020) T75

coated flasks (Corning, catalog number No.:430641) and cultured in DMEM/F12 (Millipore, catalog number DF-042-B) with B27 supplement (Invitrogen, catalog number 17504044), 1% penicillin/streptomycin and FGF-2 (Millipore, catalog number GF003) according to manufacturer's instruction. When the cells were 80% confluent, they were detached using Accutase® (Millipore, catalog number SCR005) and quantitated using a Guava® instrument. For differentiation assay, the cells were seeded at 20,000 cells/cm² onto coated 6-well plates (Millipore, catalog number PICL06P05).

5 **Co-culture assay.**

10 [0421] For the co-culture assay, a 6-well transwell (Corning catalog number 3450) system was used. The adult rat hippocampal neural stem cells were plated either alone or with hUTC at 20,000 cells/cm² onto the bottom of the well. The cultures were incubated with DMEM/F12 with B27 supplement and without FGF; half of the medium was replaced with fresh medium every other day.

15 **RNA/Protein Extraction.**

20 [0422] RNA and protein were prepared using the AllPrep RNA/Protein Kit (Qiagen, Catalog number 80404) according to manufacturer's instructions. The lysis buffer used for the cells grown in the 6-well plates was scaled up accordingly.

25 **cDNA synthesis and genomic DNA removal.**

20 [0423] The procedure was isolation of mRNA and subsequent preparation of cDNA with the QuantiTect® Reverse Transcription Kit (Qiagen, catalog number 205313). Genomic DNA removal was performed according to manufacturer's instruction prior to cDNA synthesis. cDNA synthesis was performed with 0.5 micrograms of total RNA isolated from adult rat hippocampal neural stem cells using QuantiTect® RT Kit in a total volume of 20 microliters according to manufacturer's instruction.

Quantitative TaqMan PCR.

30 [0424] PCR was performed in ABI 7000 Real time PCR System in optical 96-well reaction plates (Applied Biosystems, catalog number 4306737) in a final volume of 20 microliters. Rodent transcripts were detected with a reaction mixture containing 10 microliter 2x TaqMan universal PCR master mix (Applied Biosystems, catalog number 4364338), 1 microliter - 20X TaqMan gene expression assay (Applied Biosystems, catalog number 4331182); GFAP (assay ID: Rn 00566603_m1), nestin (Rn00364394_R1), myelin basic protein (assay ID: Rn00566745_m1), Sox2 (rn00584808_m1), tubulin beta III isoform (assay ID: Rn 00594933_m1) and GAPDH (assay ID: 99999916_s1). 1 microliter of template DNA and 8 microliter RNase-free water (Sigma, catalog number W4502) were prepared. Amplifications were performed starting with a UNG activation step at 50°C for 2 minutes followed by a 10-minute template denaturation step at 95°C. 40 cycles of denaturation at 95°C for 15 seconds and combined primer annealing/extension at 60°C for 1 minute were performed.

40 **Protein Immunoblotting.**

45 [0425] Whole cell lysates (20 micrograms) were denatured by boiling in Laemmli buffer (Boston Bioproducts, catalog number BP-111R) for 3 minutes. Subsequently, the samples then resolved by SDS-PAGE on a 10% Tris-Glycine gel (Invitrogen, catalog number: EC6078BOX) in Tris glycine SDS running buffer (Boston Bioproducts, catalog number BP-150) at 40 mA for 1 hour. The proteins were transferred onto a 0.45 micron PVDF membrane (Invitrogen, catalog number LC2005) using a semi-dry electrophoretic Trans-Blot® Cell (Bio-Rad) in transfer buffer (25 mM Tris-Base; 192 mM glycine; 10% (v/v) methanol). The transfer was performed at 25 V for 45 minutes. The membrane was blocked with 3% (w/v) BSA in PBST (PBS plus 0.1% (v/v) Tween 20) at room temperature for 1 hour. Blots were probed with primary antibodies for 1 hour at room temperature. Thereafter, the membranes were washed 3 times with PBST and subsequently incubated with secondary antibodies conjugated with horseradish peroxidase for 1 hour at room temperature at 1:5000 in PBS. Signals were detected using the SuperSignal® West Dura extended duration substrate (Pierce, catalog number 34076). The following antibodies were used: anti-tubulin III beta isoform (1:250, Millipore, Catalog number MAB 1637); anti-GFAP (1:2000, DAKO, catalog number Z0334); anti-Sox (1:1000, Abcam, catalog number ab59776); anti-MBP (1:500, Millipore, catalog number MAB 382) anti-GAPDH peroxidase conjugate (1:5000, Sigma, catalog number G9295); goat anti-mouse IgG HRP (R&D systems, catalog number HAF007); and goat anti-rabbit IgG HRP (Sigma, catalog number A0545).

Flow Cytometry.

[0426] Adult Rat Hippocampal Neural Stem Cells were detached with Accutase® after co-culture assay and briefly suspended in PBS containing 1% paraformaldehyde. Cells were then resuspended and incubated with blocking buffer (PBS with 0.3% Triton X-100 (Sigma, catalog number T9284)) and 0.3% goat serum (Millipore, catalog number S26) for 15 to 30 minutes followed by primary antibodies (1:200, except for GFAP at 1:2000) in blocking buffer for 1 hour at room temperature. Subsequently, the cells were washed three times with buffer (PBS with 0.3% Triton-X100) prior incubation with the appropriate fluorophore-conjugated secondary antibodies (1:400), such as e.g. R-phycocerythrin-conjugated goat anti-rabbit IgG (Molecular Probes, catalog number P-2771MP) and Alexa Fluor® 488-conjugated goat anti-mouse IgG (Molecular Probes, catalog number A-11029), in blocking buffer for 45 minutes. Thereafter, the stained cells were washed three times with washing buffer, suspended in PBS and subjected to analyses by using flow cytometry (FAC Scalibur™).

Immunocytochemistry.

[0427] Adult Rat Hippocampal Neural Stem Cells seeded on a 24-well plate on day 5 after co-culture assay were washed with cold PBS and fixed with 3% paraformaldehyde for 20 minutes at 4°C. Fixed cells were washed twice with PBS, followed by permeabilization with blocking buffer (room temperature, 15 minutes) and incubation at room temperature for 1 hour with primary antibodies (1:200, except for GFAP at 1:2000). Stained cells were washed three times in washing buffer before incubation with the appropriate fluorophore-conjugated secondary antibodies. After the final wash (five times in washing buffer), the stained cells were examined by confocal fluorescence microscopy (Olympus). All images were captured with a 20X objective lens.

Results**hUTC induce differentiation of neural stem cells.**

[0428] To investigate the effect of hUTCs on neural stem cell differentiation, a co-culture experiment was performed with or without the addition of FGF-2. Without supplemented FGF-2, the cells tend to differentiate poorly. To analyze the differentiation capacity of the neural stem cells with or without hUTC after 3 days, the neuronal and non-neuronal makers were quantified by real-time PCR, Western analysis and flow cytometry. Cell phenotype was identified by measuring the expression of key marker genes: SOX for progenitor cells; glial fibrillary acidic protein (GFAP) for astrocytes; tubulin beta III for neurons and myelin basic proteins (MBP) for oligodendrocytes. The results show that hUTC induced an increase in GFAP transcripts; the increase was even higher in the presence of exogenous FGF-2. The expression of tubulin beta III isoform was three fold higher in the presence of hUTC and FGF-2. GFAP protein level was also increased. Flow cytometry showed: (1) a clear population of cells that exhibited GFAP expression; and (2) a clear but small population of tubulin beta III positive cells. A similar protein expression was observed in the absence or presence of FGF-2 in the medium. The same experimental system was performed using neural stem cells, which were chemically injured by sodium azide and deoxyglucose. When the ischemic neural stem cells were treated with hUTC, a similar trend of transcript and protein expression was observed, except that GFAP transcripts increase 100-200 folds in the presence of FGF-2.

Neuronal and non-neuronal transcripts quantitated by real-time PCR.

[0429] Adult rat hippocampal neural stem cells were co-cultured either indirectly (Table 23-1) or directly with hUTCs (Table 23-2). The transcript level is expressed as fold-increase over the control cells (neural stem cells alone). The results are tabulated from two independent sets of experiments. Data are means of \pm S.D.

Table 23-1: Neuronal and non-neuronal transcripts quantitated by real-time PCR for Indirect co-culture

	Control (untreated)	Co-culture with hUTC (in the presence of exogenous FGF)	Co-culture with hUTC (in absence of exogenous FGF)
Sox (progenitor)	1	1.03 \pm 0.23	2.11 \pm 1.1
GFAP (astrocytes)	1	57.68 \pm 1.41	12.63 \pm 1.15
Neurons (tubulin beta, isoform III)	1	3.01 \pm 1.5	1.14 \pm 0.65

(continued)

Table 23-1: Neuronal and non-neuronal transcripts quantitated by real-time PCR for Indirect co-culture

	Control (untreated)	Co-culture with hUTC (in the presence of exogenous FGF)	Co-culture with hUTC (in absence of exogenous FGF)
Myelin basic protein (oligodendrocytes)	1	0.93 ± 0.04	0.625 ± 0.15
Data expressed as fold increase over controls			

Table 23-2: Neuronal and non-neuronal transcripts quantitated by real-time PCR for direct co-culture

	Control (untreated)	Co-Culture with hUTC (in absence of exogenous FGF)
Sox (progenitor)	1	0.83 ± 0.26
GFAP (astrocytes)	1	50.63 ± 7.1
Neurons (tubulin beta, isoform III)	1	3.14 ± 2.66
Myelin basic protein (oligodendrocytes)	1	0.16 ± 0.11

Data expressed as fold increase over controls

[0430] Summary. Factors secreted by hUTCs promoted the differentiation of neural stem cells to glial and neuronal *in vitro*.

EXAMPLE 24

Trophic Factors for Neural Progenitor Differentiation using PC12 cells as a model

[0431] PC 12 cells are a useful model system for neuronal differentiation. These cells proliferate indefinitely in culture but can differentiate into neuron-like cells when treated with specific factors, including nerve growth factor (NGF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and possibly interleukin 6 (IL-6), granulocyte colony-stimulating factor (GCSF) and Leukemia inhibitory factor (LIF). Previous studies have indicated that hUTC secrete neurotrophic factors *in vitro*. The aim of the present study was to determine if soluble factors from hUTC could promote the differentiation of PC12 cells.

Materials and Methods

Cell culture and conditioned medium.

[0432] Four million human umbilical tissue-derived cells (hUTC) were thawed (Lot no. 1027, passage 2 or 4), seeded onto T75 flasks and cultured in Hayflick growth medium (DMEM - low glucose (Gibco, Catalog number 11885-084), 15% v/v fetal bovine serum (FBS, Hyclone catalog number SH30070.031R), 4mM Glutamax (Gibco, catalog number 3505-061) and 1% penicillin/streptomycin (Gibco catalog number 1070063) overnight. The following day, the cells were washed three times with phosphate buffered saline (PBS, Invitrogen, catalog number 25200-056) and incubated with Roswell Park Memorial Institute 1640 (RPMI, ATCC, catalog number 30-2001), 0.5% heat inactivated horse serum (Gibco, catalog number 26050088) and 1% penicillin/streptomycin for 48 hours. The medium was removed and centrifuged at 2,500 rpm for 5 minutes.

[0433] Neuroscreen-1 cells, subclone of PC12 cells (Lot no. NS1081219, Cellomics, catalog number R04-0001-C1) were thawed (P2 or P6), seeded onto collagen I coated flasks (Nunc, Catalog number 132707) and cultured in RPMI with 10% heat inactivated horse serum, 5% FBS (ATCC, catalog number 30-2020), and 1% penicillin/streptomycin according to manufacturer's instruction. When the cells were 70% confluent, they were detached using TrypLE™ (Gibco,

Catalog number 12604-021) and quantitated using a Guava® instrument. For differentiation assay, the cells were seeded at 20 to 30,000 cells/cm² in collagen coated 6-well plates (Millipore, catalog number PICL06P05).

Treatment with growth factors and conditioned medium.

5

[0434] Neuroscreen-1 cells were washed three times with PBS and they were quiescent for 18-24 hours with RPMI and 0.5% horse serum before treatment with 10 ng/ml human nerve growth factor beta (NGF, Millipore, catalog number GF028) or conditioned medium. The cells were cultured for 4 days.

10

Neurite outgrowth assay.

15

[0435] Immunocytochemical evaluation of neurite outgrowth was performed using a Neurite Outgrowth kit according to manufacturer's instructions (Thermo Fisher Scientific). After treatment with NGF or hUTC conditioned medium at the stated time point, cells were fixed for 20 minutes using 4% paraformaldehyde in PBS at 37°C. Cell bodies and processes were labeled using an anti-beta III tubulin primary antibody, followed by an Alexa Fluor® 488-conjugated secondary antibody. Hoechst 33258 dye was included in the fixative to label cell nuclei. Plates were then loaded into a Cellomics ArrayScan® VTI high content imaging platform for automated image capture and analysis. This system is based on an inverted epifluorescence microscope that automatically focuses and scans fields in individual wells using a motorized stage. Fluorescence images were produced using a multiple bandpass emission filter and matched excitation filters for the blue channel (nuclei) and green channel (cell body and processes) and acquired with a high-resolution charge-coupled device camera. The acquired images were analyzed by the ArrayScan® software using the Neuronal Profiling bioapplication (Thermo Fisher Scientific) to measure various morphological parameters including total neurite length per cell, neurites per cell, branch points and cell body area for each valid cell in the image. A cell was defined as differentiated when it had a total neurite length > 5 micrometer, which is twice the total neurite length in cells in the absence of NGF. Using a 10× objective, a sufficient number of fields were acquired for the analysis of at least 500 cells per well.

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Results

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[0436] To assess whether hUTCs induce neurogenesis in neuroscreen-1 cells, conditioned medium (from cultured hUTCs) were used to culture quiescent PC12 cells for 4 days. For the positive control, neuroscreen-1 cells were treated with 10 ng/ml NGF. The effect of NGF or conditioned medium was observed by microscopy. In the absence of NGF or hUTC conditioned medium, cells are relatively small and rounded and have very few visible neurites. Treatment with NGF or conditioned medium for 2 to 4 days resulted in slightly larger cell bodies with an extension network of neurites. There was a gradual increase in neurite length after exposure to NGF or conditioned medium. More than 40% of the cells were differentiated on the fourth day after exposure to the conditioned medium. To quantify neuroscreen-1 cell differentiation, cells were labeled with beta-tubulin isoform III antibodies; images were acquired and analyzed by the Neuronal Profiling BioApplication. In both NGF and hUTC conditioned medium treated PC 12 cells, there were increases in the neurite length, number of neurites and branch points per cells. Thus, hUTC conditioned medium is sufficient to induce PC 12 cells differentiation.

40

Neuroscreen-1 cells induced by NGF and hUTC 48 hour conditioned medium (Table 24-1).

45

[0437] Neuroscreen-1 cells were grown on 12-well plates for 4 days in 0.5% horse serum RPMI (negative control), or with 20ng/ml NGF (positive control) or 48 hour conditioned medium. After 4 days, cells were fixed and stained as described in Materials and Methods. The results are tabulated from two independent sets of experiments. Data are means of \pm S.D.

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Table 24-1: Neuroscreen-1 cells induced by NGF and hUTC 48 hour conditioned medium

	Neurite total length per neuron (μ m)	Branch point total count per 500 cells	Neurite outgrowth index (%)
Negative Control	2.45 \pm 1.32	7.48 \pm 4.77	8.03 \pm 5.53
Positive Control (20 ng/ml NGF)	9.83 \pm 1.75	34.46 \pm 1.68	32.73 \pm 6.73

(continued)

Table 24-1: Neuroscreen-1 cells induced by NGF and hUTC 48 hour conditioned medium

	Neurite total length per neuron (μm)	Branch point total count per 500 cells	Neurite outgrowth index (%)
hUTC 48 hr conditioned medium	16.44 \pm 0.11	68.27 \pm 19	41.4 \pm 1.59

10 [0438] **Summary:** Results indicated that soluble factors secreted by hUTCs induce neurogenesis in PC 12 cells as indicated by morphological changes

EXAMPLE 25

15 Prophetic protocol for Multiple Administration of hUTC for Amyotrophic Lateral Sclerosis

10 [0439] The study results suggest that hUTCs may be efficacious in the SOD1 G93A rat model of Amyotrophic Lateral Sclerosis (ALS) with a single administration. As such, additional studies may demonstrate that repeat administration of hUTCs may have added efficacy in this disease model.

20 [0440] SOD1 G93A may be treated with hUTCs at or near the time of disease onset with hUTC injected directly into the intrathecal space or cisterna magna. Cells may be injected weekly or more or less frequently. To minimize injury and maximize prompt recovery of the animal, a transcutaneous injection method to intrachecally deliver the hUTC cell product into the CSF of the lumbar cistern of SOD1 rats may be used. Animals are mounted on Kopf stereotaxic device and anesthetized with gas anesthesia (isoflurane: oxygen: nitrous oxide = 1:33:66). The lumbar region is shaved and a cylindrical support element is placed under the rat lower abdomen to stretch the intervertebral spaces of the lumbar spine. Cells are delivered with 200-500 μl microsyringes fitted with a 25Gx1" needle. The needle with empty microsyringe is slowly inserted into the subarachnoid space at the L4-5 or L6-S1 intervertebral spaces. When a physical sign of irritation of the cauda equina is obtained, *i.e.* tail or hind paw flick, cells are slowly infused into the subarachnoid space over 1 minute period. Syringe is held in place for 2 minutes and then removed. The needle entry point is pressed with a cotton swab tip immersed in iodine for 1 minute, and then the animal is dismounted and resuscitated on a heat pad. After the animal wakes up, it is returned to its cage.

30 [0441] Animals may be treated with hUTCs at different dose levels with delivery of a constant amount of total injection volume. Animals may be treated with hUTC at approximately biweekly intervals and evaluated to end-stage.

Table 25-1: Design study to evaluate multiple hUTC injections

Group Number	Number of Animals	Test Material	Dose Number/Dosage	Dose Volume (μl)	Route of Administration	Time to be euthanized
1	15	Vehicle	4/0	50	IT	End-stage
2	15	hUTC	1/0	50	IT	End-stage
3	15	hUTC	4/0.3 \times 10 ⁶ Cells	50	IT	End-stage
4	15	hUTC	4/1 \times 10 ⁶ Cells	50	IT	End-stage
5	15	Riluzole	Per day/44mg/kg	2 ml	Oral	End-stage
6	15	Vehicle	2/0	50	IT	Midpoint
7	15	hUTC	2/1 \times 10 ⁶ Cells	50	IT	Midpoint

55 [0442] The disease progression and potential therapeutic efficacy are monitored through two outcome measurements: (1) functional locomotor activity (measured weekly); and (2) body weight (measured twice a week). The locomotor tests include the Basso, Beattie and Bresnahan (BBB) test (Basso et al. (1995) J Neurotrauma 1995; 12(1): 1; Basso et al. (1996) Exp. Neurol. 139: 244-256) and the inclined plane performance test (Rivlin AS, Tator CH. (1977) J Neurosurg 47(4): 577).

[0443] For BBB scoring, the rats are gentled and adapted to the open field. Once a rat walks continuously in the open field, two examiners, blinded for the treatment, conduct a 4-minute testing session using the BBB locomotor rating scale

that is a 21-point scale with high intra- and interobserver reproducibility. The open field test is videotaped. From this tape, two examiners quantitatively assessed the number of hindlimb (HL) movements performed during a 1-minute period; this period is a composite of episodes over the 4-minute testing session when each HL is in full view of the camera. The 1-minute time limit ensures that the right and left HLs of each animal are assessed for the same length of time since both HLs are not always in full view of the camera. The footage is reassessed in slow motion if the examiners' scores disagree. Absence of observable hind limb movement is scored 0 and the initial points were awarded for isolated joint movements. The score is increased when more joints show movement and/or the movements are more extensive. As locomotion increases, points are given for plantar placement of the paw, weight support and coordination between forelimb and hind limb. The final points are achieved by toe clearance, trunk stability and tail position.

[0444] The inclined plane is another behavioral task that assesses the animal's ability to maintain its position on a rubber corrugated board; this board was raised at 5° increments. The maximum angle at which an animal can support its weight for 5 seconds is defined as the capacity angle. This test examines sensory feedback, coordination, and muscle strength required for locomotion.

[0445] The end-stage of the disease is determined by paralysis so severe that the animal could not right itself within 20 seconds when placed on its side, which is an endpoint frequently used for SOD1 mutant rats. Both BBB and inclined plane scores at this point are zero,

[0446] The present invention is not limited to the embodiments described and exemplified above. It is capable of variation and modification within the scope of the appended claims.

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[0447]

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25 Mistry, Sanjay

Harmon, Alexander M.

Harris, Ian Ross

Kihm, Anthony J.

Seyda, Agnieszka

30 Yi, chin-Feng

Gosiewska, Anna

<120> REGENERATION AND REPAIR OF NEURAL TISSUE USING POSTPARTUM-DERIVED CELLS

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45 **Claims**

1. Umbilical cord tissue-derived cells for use in preserving motor neuron function in a patient with amyotrophic lateral sclerosis, wherein the umbilical cord tissue-derived cells are isolatable from human umbilical cord tissue substantially free of blood, are capable of self renewal and expansion in culture, have the potential to differentiate into cells of other phenotypes, can undergo at least 40 doublings, and have the following characteristics:
 - (a) express each of CD 10, CD 13, CD44, CD73, CD90, PDGFr-alpha, PD-L2 and HLA-A,B,C;
 - (b) does not express any of CD31, CD34, CD45, CD80, CD86, CD117, CD141, CD 178, B7-H2, HLA-G, or HLA-DR,DP,DQ; and
 - (c) have increased expression of interleukin-8; reticulon 1; and chemokine receptor ligand (C-X-C motif) ligand 3, relative to that of a human cell which is a fibroblast, a mesenchymal stem cell, or an iliac crest bone marrow cell.
2. The umbilical cord tissue-derived cells for the use of claim 1, wherein the umbilical cord tissue-derived cells do not

express hTERT or telomerase.

3. The umbilical cord tissue-derived cells for the use of claim 1, wherein the umbilical cord tissue-derived cells are induced *in vitro* to differentiate into a neural cell line prior to administration.
5. The umbilical cord tissue-derived cells for the use of claim 1, wherein the preserving comprises administering the umbilical cord tissue-derived cells with at least one other cell type of an astrocyte, oligodendrocyte, neuron, neural progenitor, neural stem cell or other multipotent or pluripotent stem cell.
10. The umbilical cord tissue-derived cells for the use of claim 1, wherein the preserving comprises administering the umbilical cord tissue-derived cells at a pre-determined site in the nervous system of the patient.
15. The umbilical cord tissue-derived cells for the use of claim 1, wherein the preserving comprises administering the umbilical cord tissue-derived cells by injection or infusion.
20. The umbilical cord tissue-derived cells for the use of claim 1, wherein the preserving comprises administering the umbilical cord tissue-derived cells by intravenous or intrathecal injection.
25. The umbilical cord tissue-derived cells for the use of claim 1, wherein the preserving comprises administering the umbilical cord tissue-derived cells encapsulated within an implantable device
30. The umbilical cord tissue-derived cells for the use of claim 1, wherein the preserving comprises administering the umbilical cord tissue-derived cells by implantation of a matrix or scaffold containing the cells.
35. The umbilical cord tissue-derived cells for the use of claim 1, wherein the umbilical cord tissue-derived cells exert a trophic effect on the nervous system of the patient.
40. The umbilical cord tissue-derived cells for the use of claim 1, wherein the umbilical cord tissue-derived cells are genetically engineered to produce a gene product that promotes treatment of the neurodegenerative condition.

Patentansprüche

1. Aus Nabelschnurgewebe abgeleitete Zellen zur Verwendung in der Aufrechterhaltung der Motoneuronfunktion in einem Patienten mit amyotropher Lateralsklerose, wobei die aus Nabelschnurgewebe abgeleiteten Zellen aus im Wesentlichen blutfreiem menschlichem Nabelschnurgewebe isolierbar sind, zur Selbsterneuerung und Expansion in Kultur fähig sind, das Potential aufweisen, sich zu Zellen anderer Phänotypen zu differenzieren, mindestens 40 Verdopplungen durchlaufen können und die folgenden Eigenschaften aufweisen:
 40. (a) jedes von CD10, CD13, CD44, CD73, CD90, PDGFr-alpha, PD-L2 und HLA-A,B,C exprimieren;
 - (b) keines von CD31, CD34, CD45, CD80, CD86, CD117, CD141, CD178, B7-H2, HLA-G oder HLA-DR,DP,DQ exprimieren; und
 - (c) erhöhte Expression von Interleukin-8; Reticulon 1; und Chemokinrezeptorligand (C-X-C-Motiv) Ligand 3 im Vergleich zu derjenigen einer menschlichen Zelle, die eine Fibroblastenzelle, eine mesenchymale Stammzelle oder eine Knochenmarkzelle des Beckenkamms ist, aufweisen.
2. Aus Nabelschnurgewebe abgeleitete Zellen zur Verwendung nach Anspruch 1, wobei die aus Nabelschnurgewebe abgeleiteten Zellen nicht hTERT oder Telomerase exprimieren.
50. 3. Aus Nabelschnurgewebe abgeleitete Zellen zur Verwendung nach Anspruch 1, wobei die aus Nabelschnurgewebe abgeleiteten Zellen vor der Verabreichung *in vitro* induziert werden, um sich zu einer Nervenzelllinie zu differenzieren.
4. Aus Nabelschnurgewebe abgeleitete Zellen zur Verwendung nach Anspruch 1, wobei das Aufrechterhalten des Verabreichen der aus Nabelschnurgewebe abgeleiteten Zellen mit mindestens einem weiteren Zelltyp eines Astrozyten, eines Oligodendrozyten, einer Nervenzelle, einer Nervenvorläuferzelle, einer Nervenstammzelle oder einer anderen multipotenten oder pluripotenten Stammzelle umfasst.
5. Aus Nabelschnurgewebe abgeleitete Zellen zur Verwendung nach Anspruch 1, wobei das Aufrechterhalten das

Verabreichen der aus Nabelschnurgewebe abgeleiteten Zellen an einer vorbestimmten Stelle im Nervensystem des Patienten umfasst.

5 6. Aus Nabelschnurgewebe abgeleitete Zellen zur Verwendung nach Anspruch 1, wobei das Aufrechterhalten das Verabreichen der aus Nabelschnurgewebe abgeleiteten Zellen mittels Injektion oder Infusion umfasst.

10 7. Aus Nabelschnurgewebe abgeleitete Zellen zur Verwendung nach Anspruch 1, wobei das Aufrechterhalten das Verabreichen der aus Nabelschnurgewebe abgeleiteten Zellen mittels intravenöser oder intrathekaler Injektion umfasst.

15 8. Aus Nabelschnurgewebe abgeleitete Zellen zur Verwendung nach Anspruch 1, wobei das Aufrechterhalten das Verabreichen der innerhalb einer implantierbaren Vorrichtung verkapselten aus Nabelschnurgewebe abgeleiteten Zellen umfasst.

20 9. Aus Nabelschnurgewebe abgeleitete Zellen zur Verwendung nach Anspruch 1, wobei das Aufrechterhalten das Verabreichen der aus Nabelschnurgewebe abgeleiteten Zellen mittels Implantation einer Matrix oder eines Gerüsts, die/das die Zellen enthält, umfasst.

25 10. Aus Nabelschnurgewebe abgeleitete Zellen zur Verwendung nach Anspruch 1, wobei die aus Nabelschnurgewebe abgeleiteten Zellen eine trophische Wirkung auf das Nervensystem des Patienten ausüben.

30 11. Aus Nabelschnurgewebe abgeleitete Zellen zur Verwendung nach Anspruch 1, wobei die aus Nabelschnurgewebe abgeleiteten Zellen so genetisch verändert worden sind, dass sie ein Genprodukt produzieren, das die Behandlung des neurodegenerativen Leidens unterstützt.

Revendications

30 1. Cellules dérivées de tissu de cordon ombilical pour utilisation dans la préservation de la fonction neuromotrice chez un patient atteint de sclérose latérale amyotrophique, où les cellules dérivées de tissu de cordon ombilical peuvent être isolées à partir de tissu de cordon ombilical humain sensiblement exempt de sang, sont capables d'autorenouvellement et d'expansion en culture, ont le potentiel de différenciation en cellules d'autres phénotypes, peuvent subir au moins 40 doublements, et possèdent les caractéristiques suivantes :

35 (a) expriment chacun de CD10, CD13, CD44, CD73, CD90, PDGFr-alpha, PD-L2 et HLA-A,B,C ;
 (b) n'expriment aucun de CD31, CD34, CD45, CD80, CD86, CD117, CD141, CD178, B7-H2, HLA-G, ou HLA-DR,DP,DQ ; et
 (c) présentent une expression accrue d'interleukine-8 ; réticulon 1 ; et ligand de récepteur de chimiokine (motif C-X-C) 3, par rapport à celle d'une cellule humaine qui est un fibroblaste, une cellule souche mésenchymateuse ou une cellule de moelle osseuse de crête iliaque.

40 2. Cellules dérivées de tissu de cordon ombilical pour l'utilisation de la revendication 1, les cellules dérivées de tissu de cordon ombilical n'exprimant pas hTERT ou la télomérase.

45 3. Cellules dérivées de tissu de cordon ombilical pour l'utilisation de la revendication 1, les cellules dérivées de tissu de cordon ombilical étant induites *in vitro* pour se différencier en une lignée cellulaire neurale avant administration.

50 4. Cellules dérivées de tissu de cordon ombilical pour l'utilisation de la revendication 1, la préservation comprenant l'administration des cellules dérivées de tissu de cordon ombilical avec au moins un autre type de cellule d'un astrocyte, un oligodendrocyte, un neurone, un progéniteur neural, une cellule souche neurale ou une autre cellule souche multipotente ou pluripotente.

55 5. Cellules dérivées de tissu de cordon ombilical pour l'utilisation de la revendication 1, la préservation comprenant l'administration des cellules dérivées de tissu de cordon ombilical à un site prédéterminé dans le système nerveux du patient.

60 6. Cellules dérivées de tissu de cordon ombilical pour l'utilisation de la revendication 1, la préservation comprenant l'administration des cellules dérivées de tissu de cordon ombilical par injection ou perfusion.

7. Cellules dérivées de tissu de cordon ombilical pour l'utilisation de la revendication 1, la préservation comprenant l'administration des cellules dérivées de tissu de cordon ombilical par injection intraveineuse ou intrathécale.
- 5 8. Cellules dérivées de tissu de cordon ombilical pour l'utilisation de la revendication 1, la préservation comprenant l'administration des cellules dérivées de tissu de cordon ombilical encapsulées dans un dispositif implantable.
9. Cellules dérivées de tissu de cordon ombilical pour l'utilisation de la revendication 1, la préservation comprenant l'administration des cellules dérivées de tissu de cordon ombilical par implantation d'une matrice ou d'un échafaudage contenant les cellules.
- 10 10. Cellules dérivées de tissu de cordon ombilical pour l'utilisation de la revendication 1, les cellules dérivées de tissu de cordon ombilical exerçant un effet trophique sur le système nerveux du patient.
- 15 11. Cellules dérivées de tissu de cordon ombilical pour l'utilisation de la revendication 1, les cellules dérivées de tissu de cordon ombilical étant génétiquement modifiées pour produire un produit génique qui stimule le traitement de l'affection neurodégénérative.

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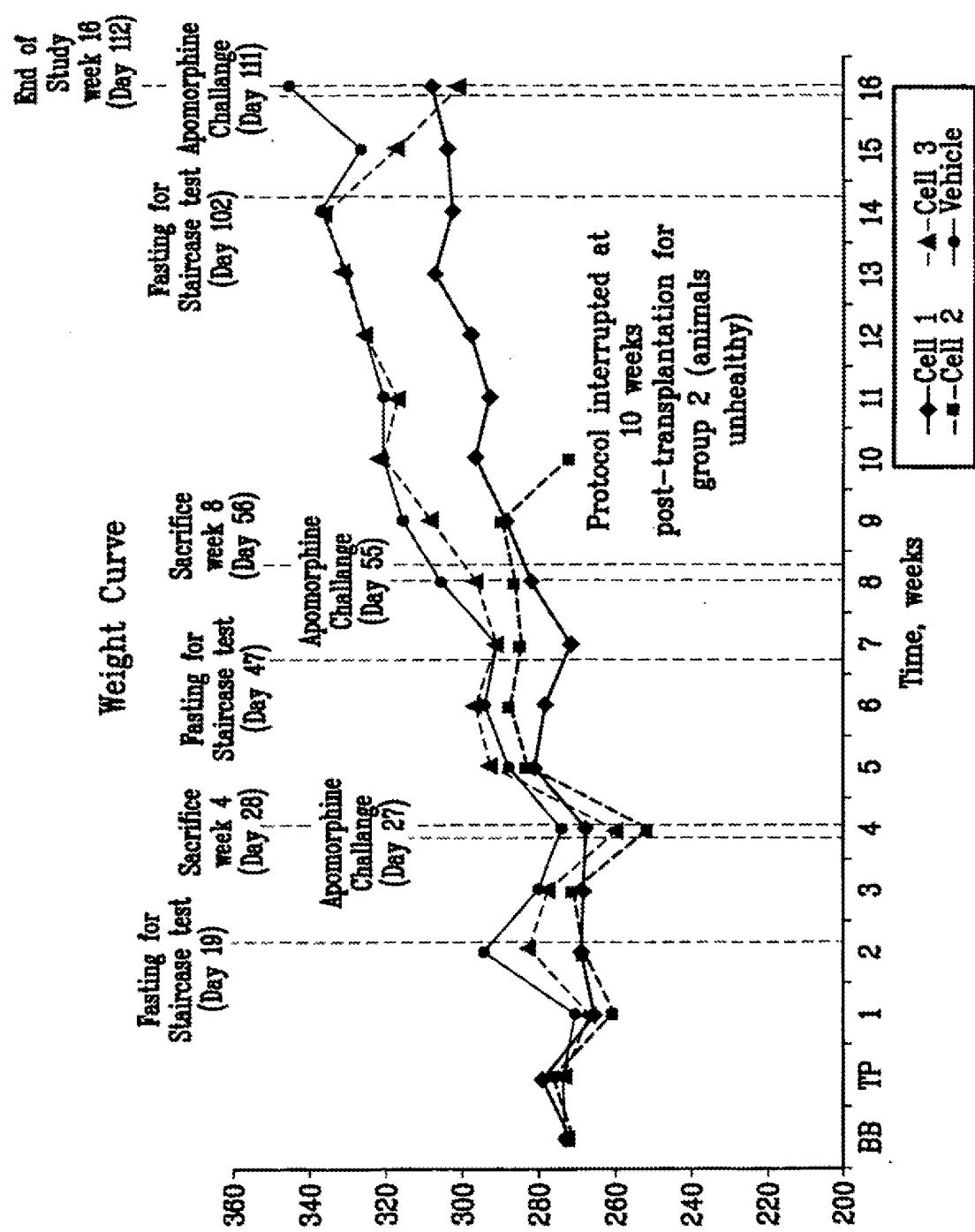


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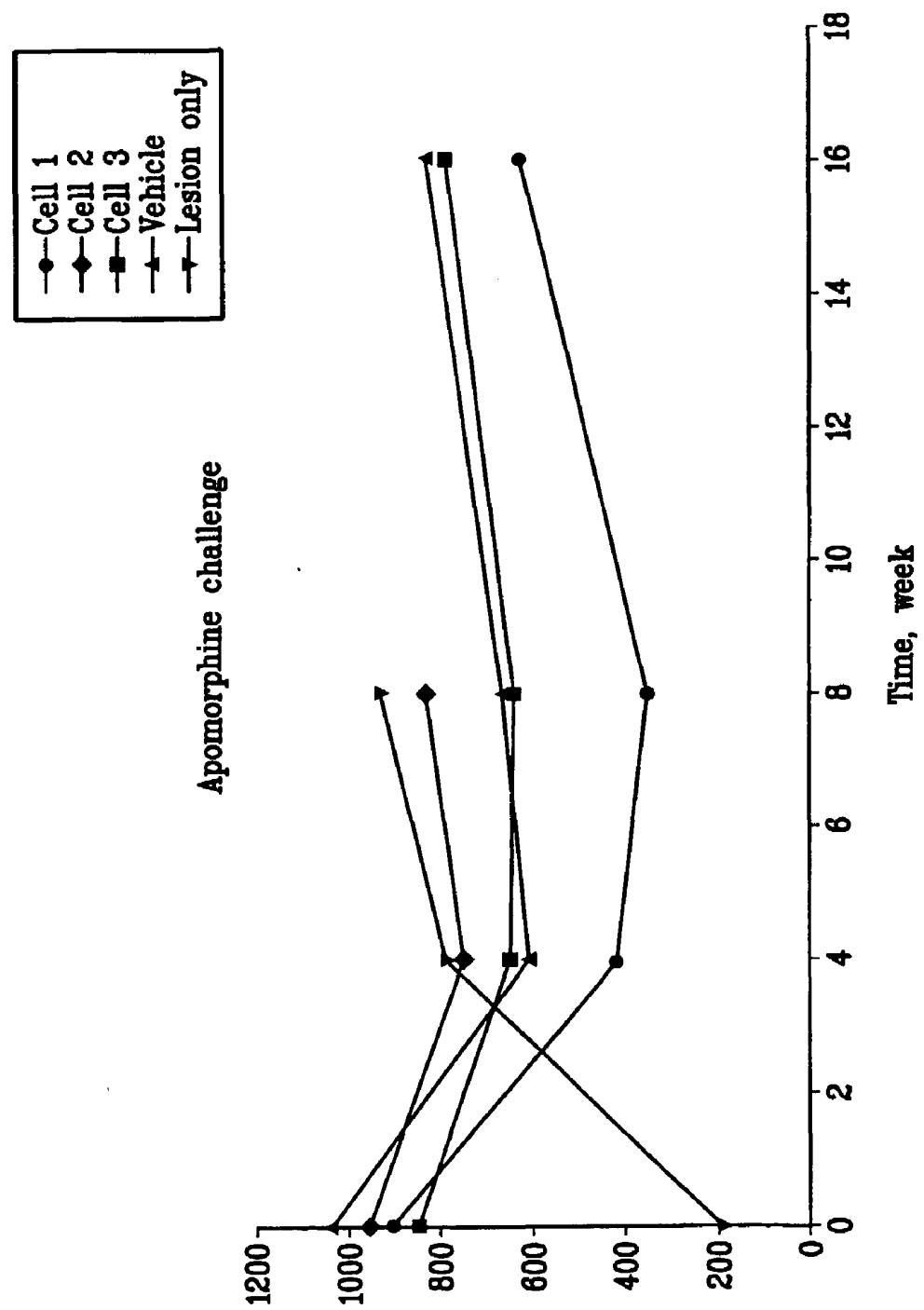


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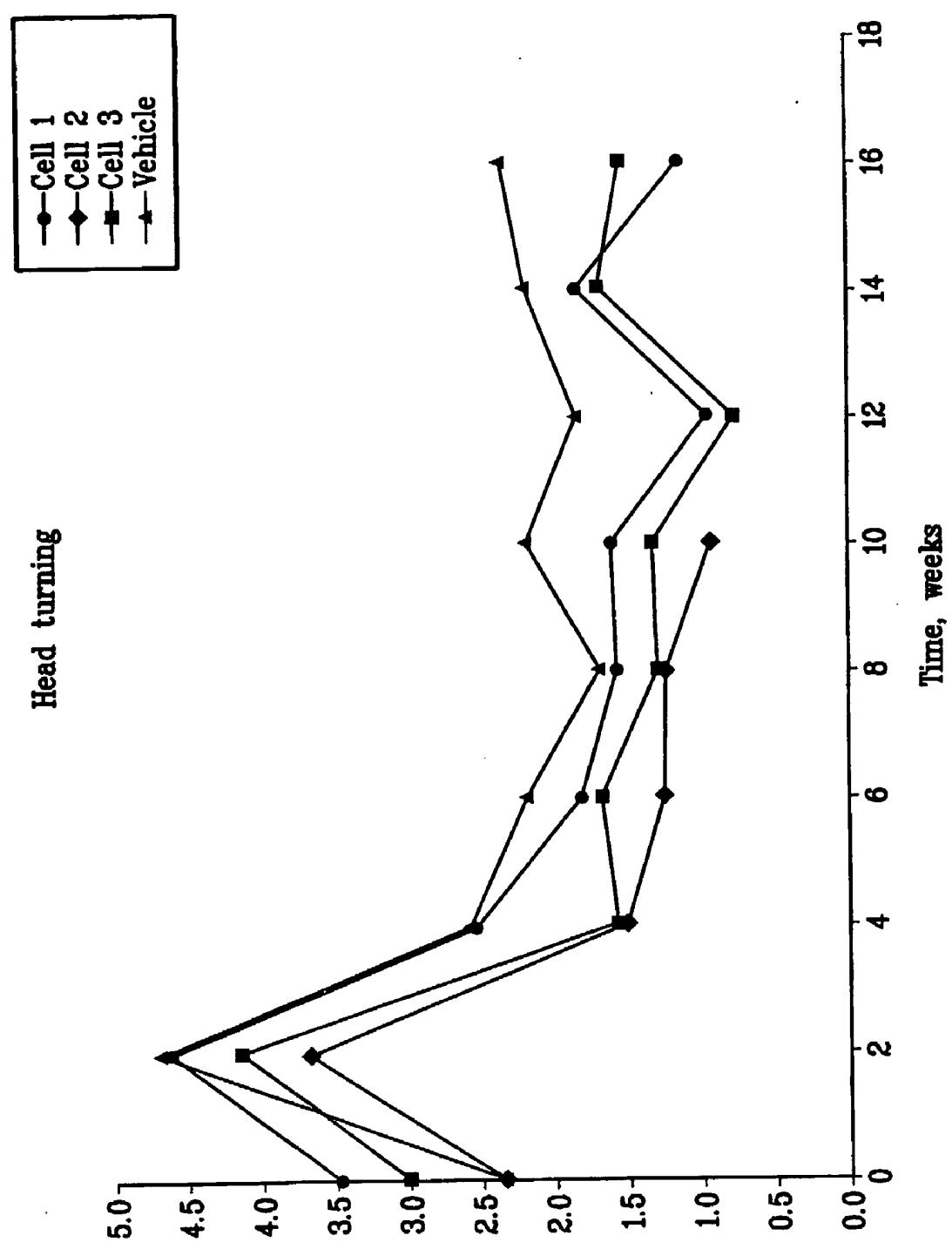


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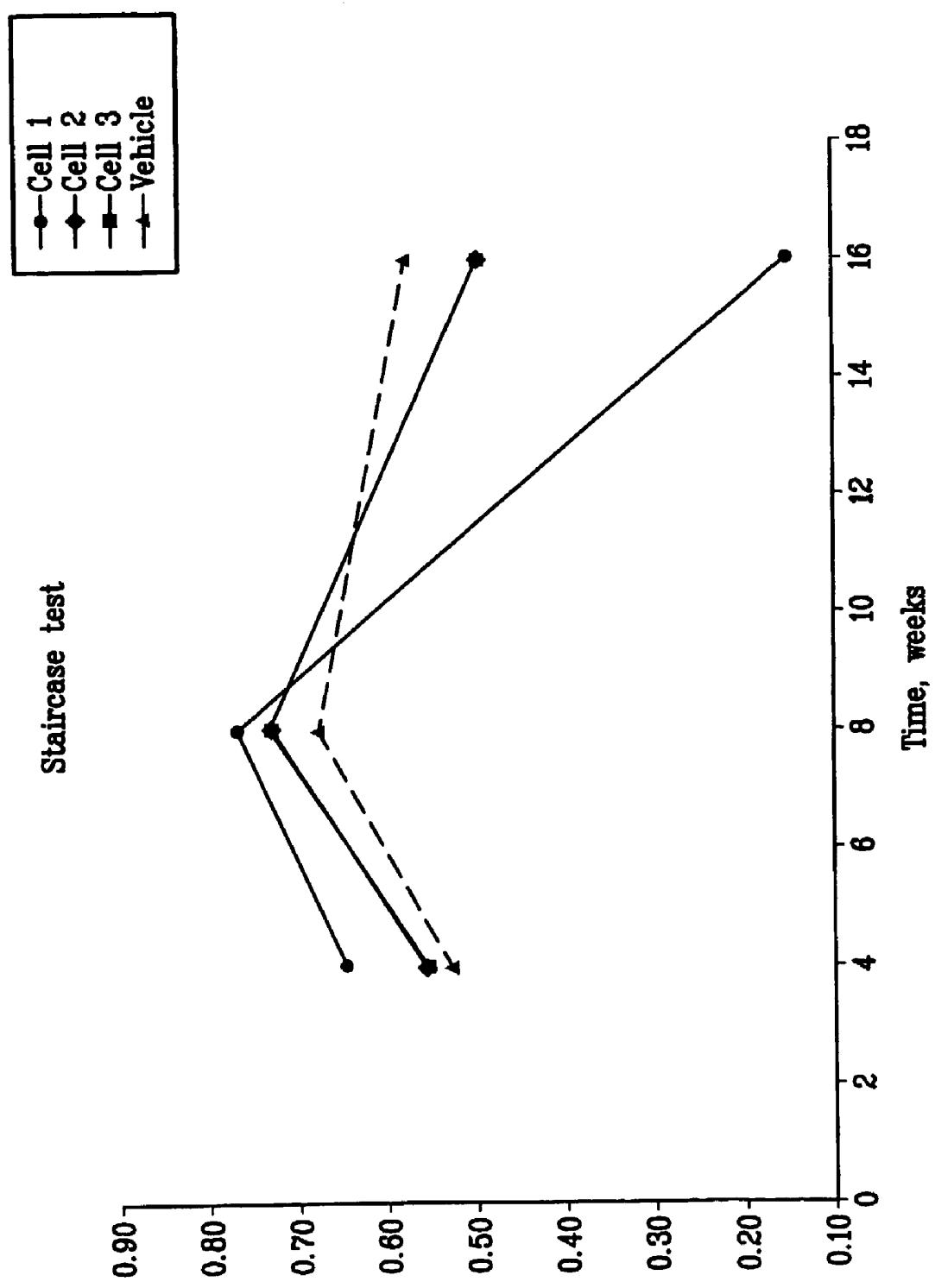
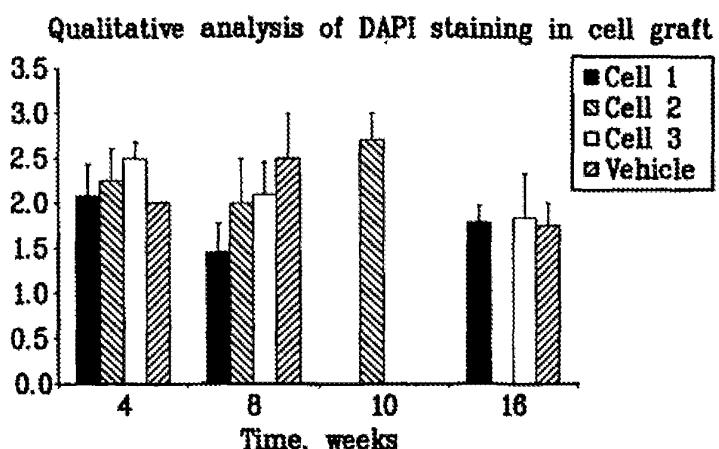
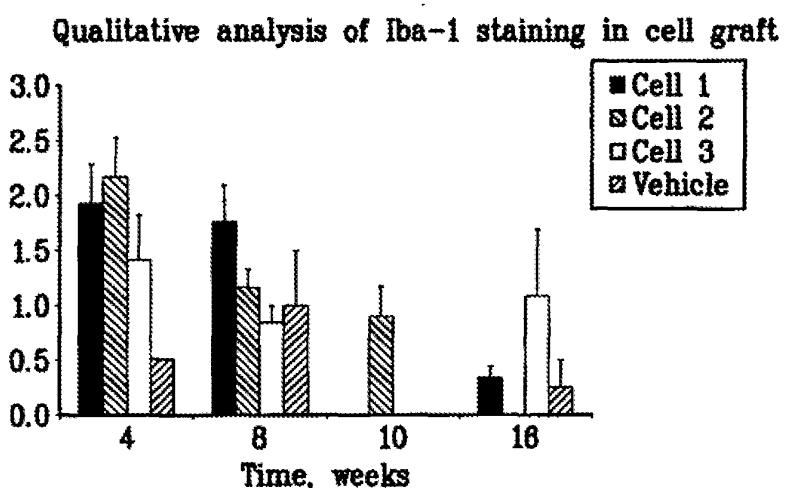


Fig. 4

A.



B.



C.

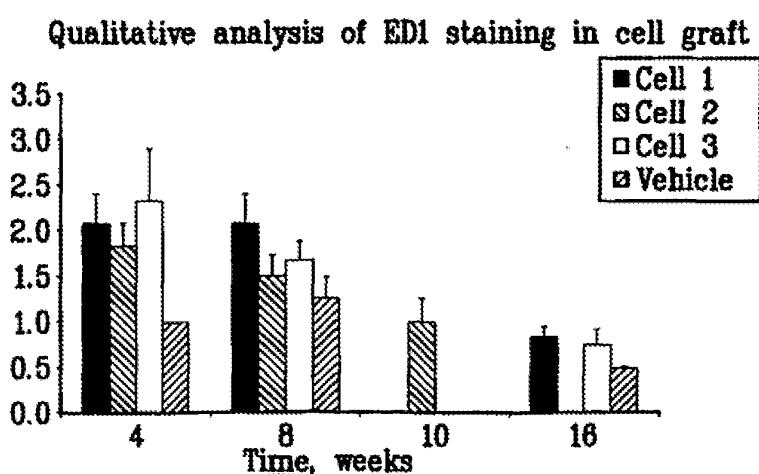
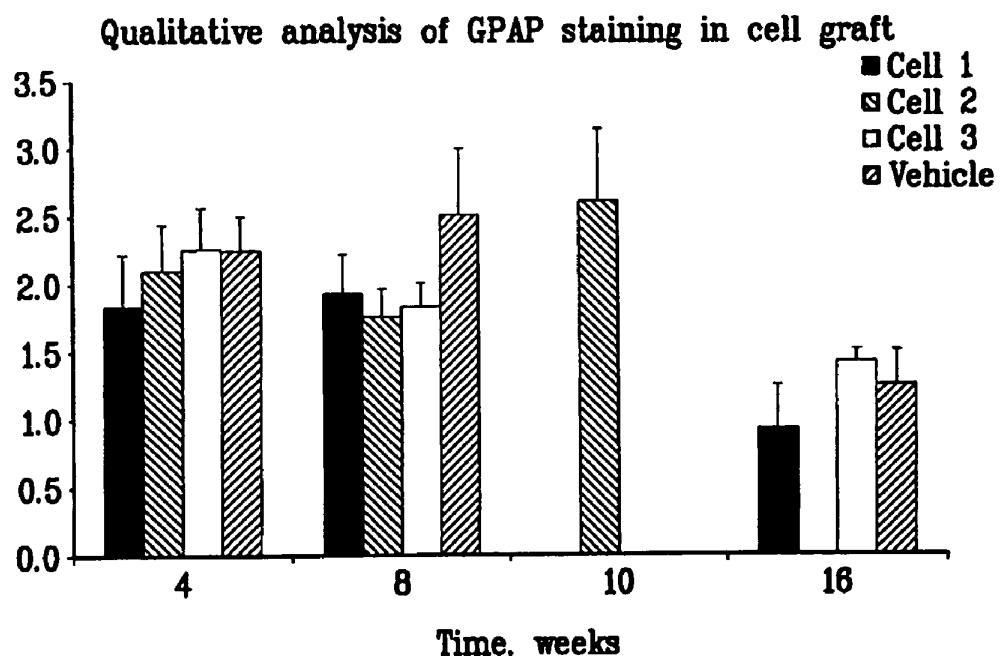


Fig. 5

A.



B.

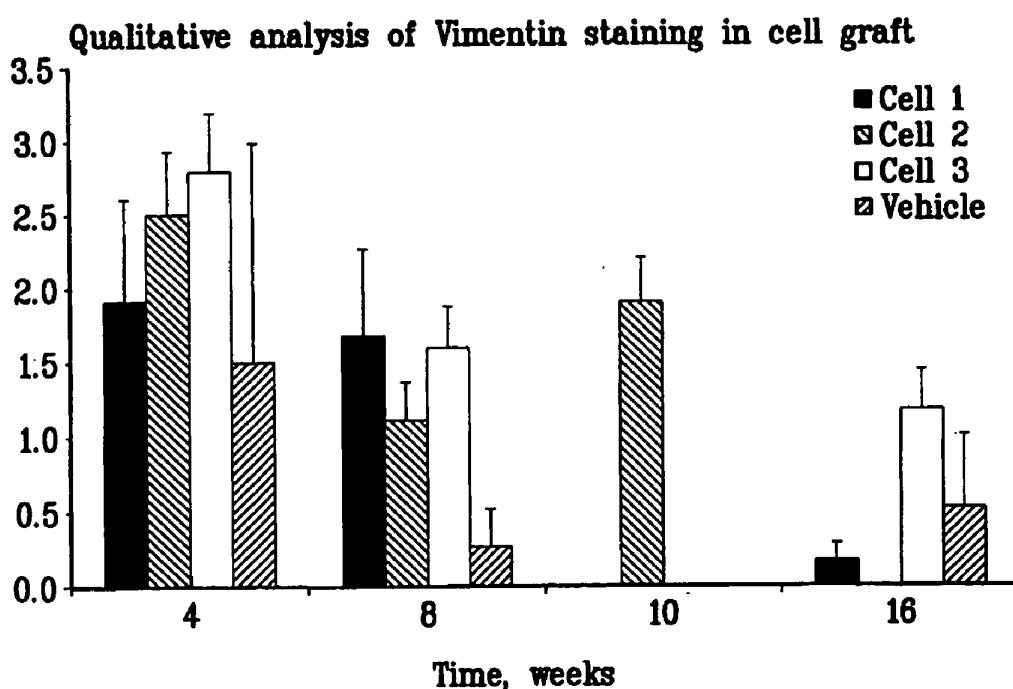
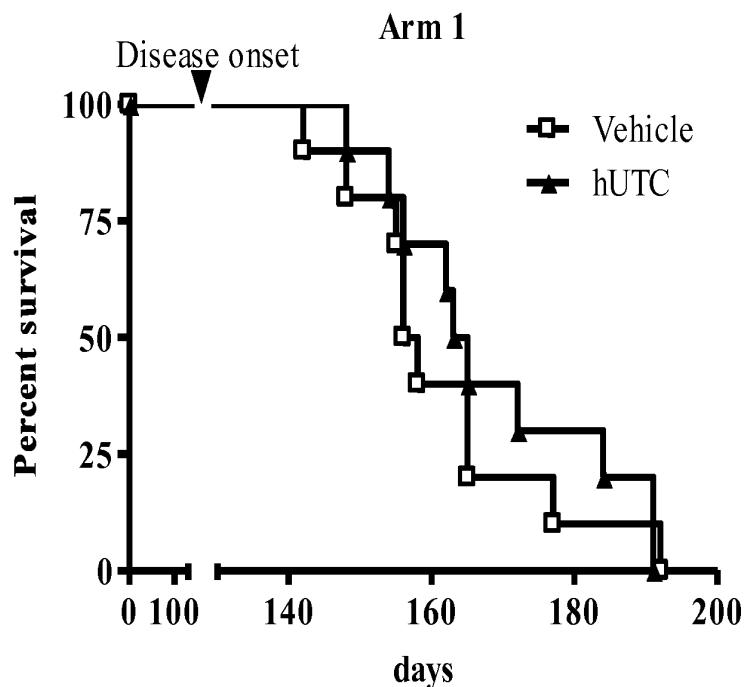
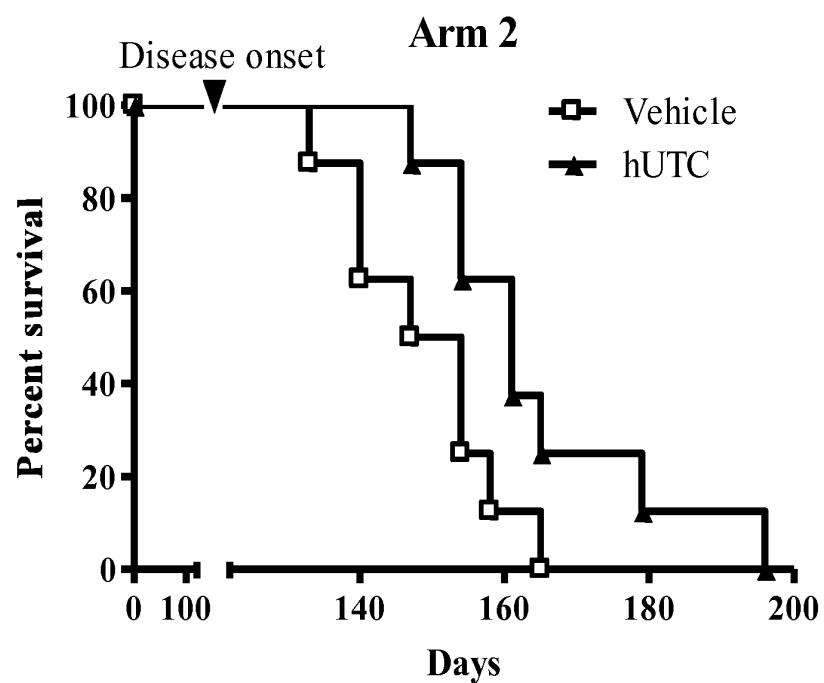


Fig. 6

A.



B.

**Fig. 7**

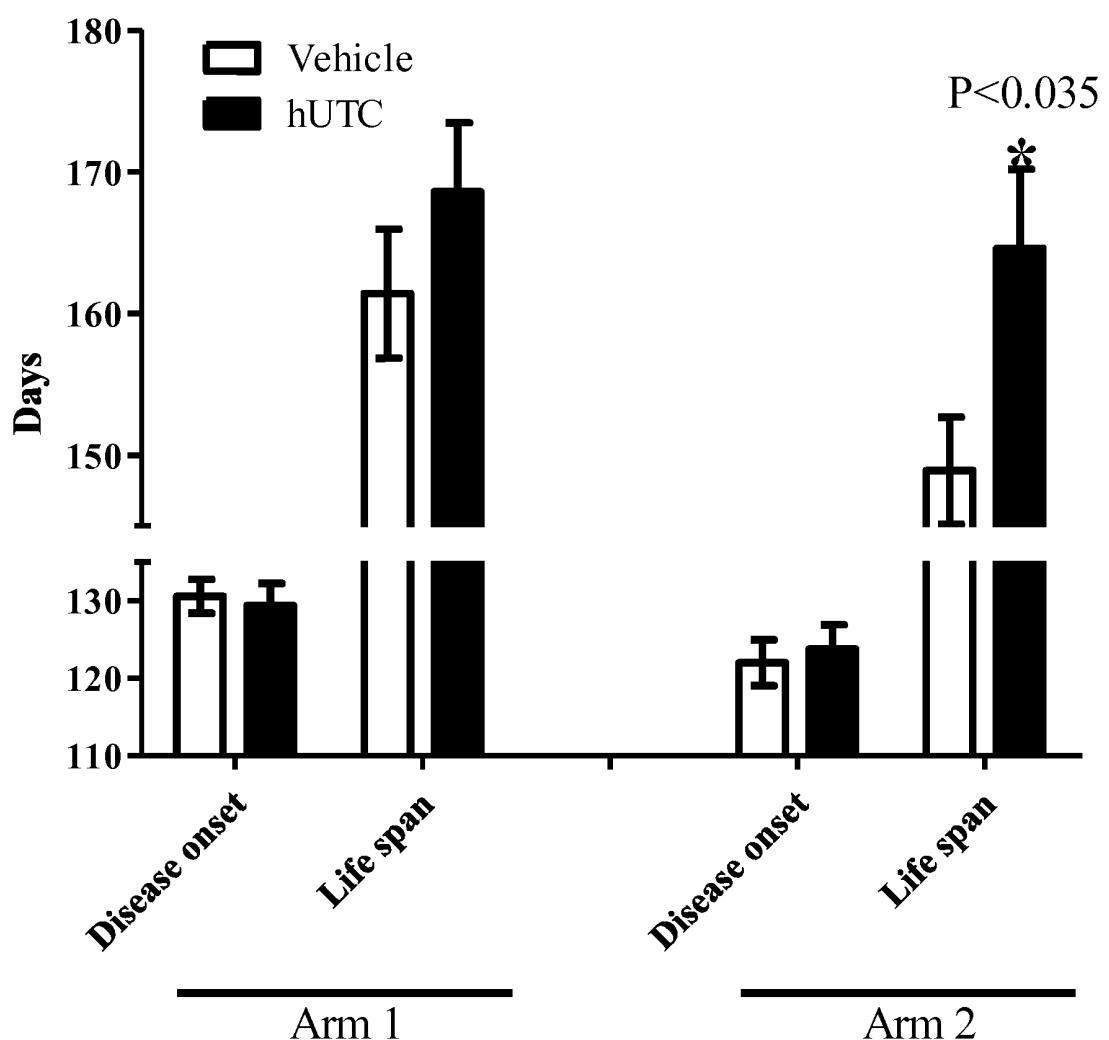
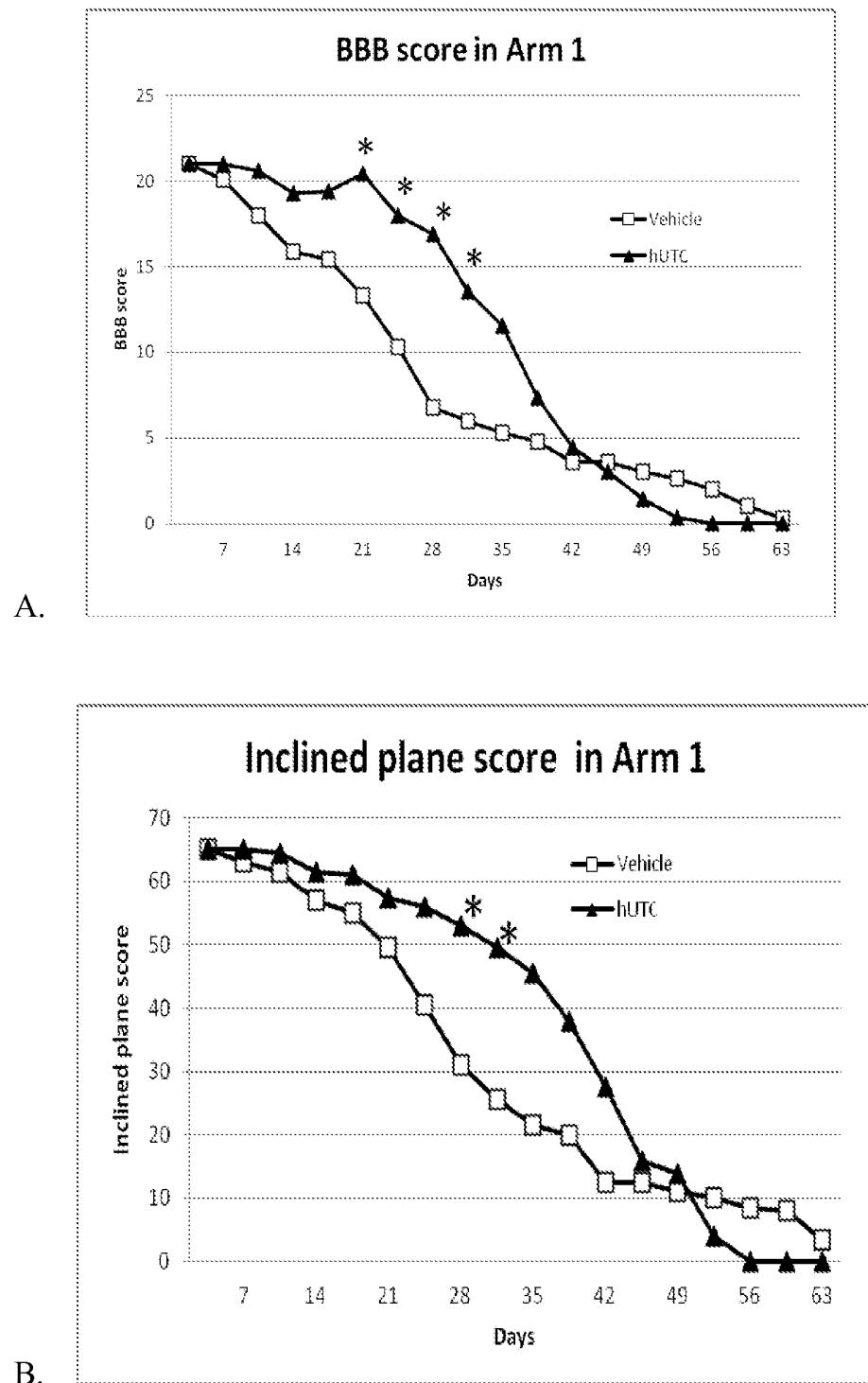
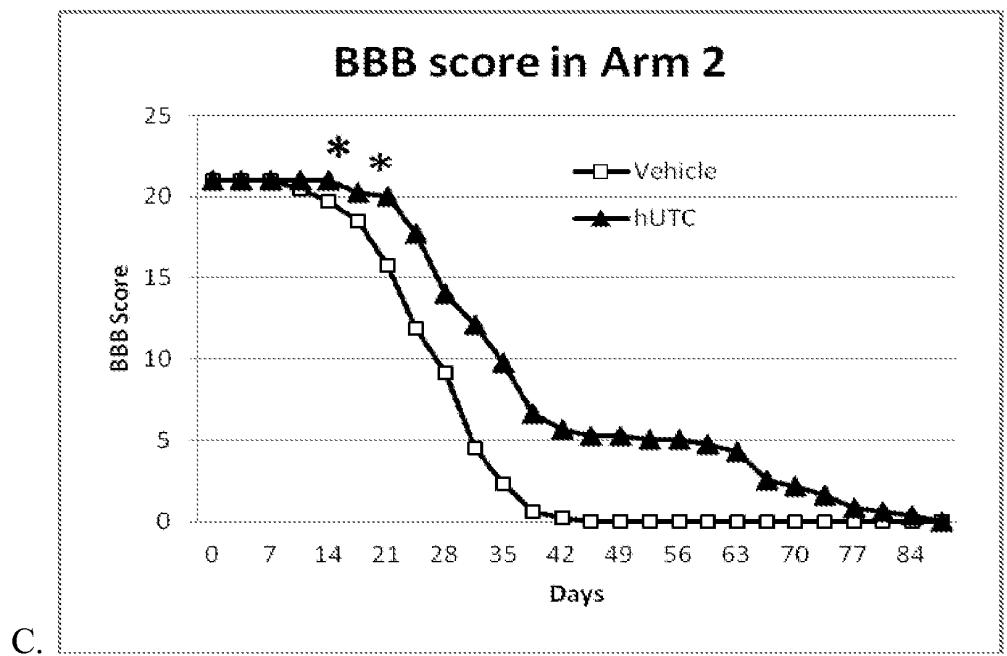
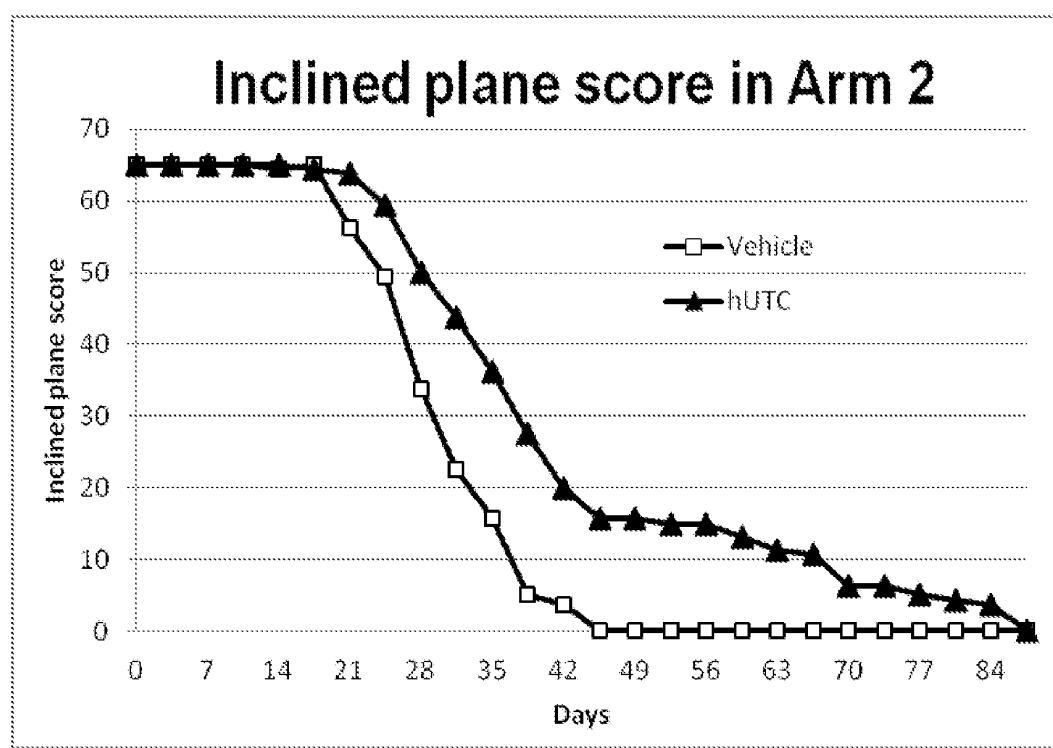


Fig. 8

**Fig. 9**



C.



D.

Fig. 9

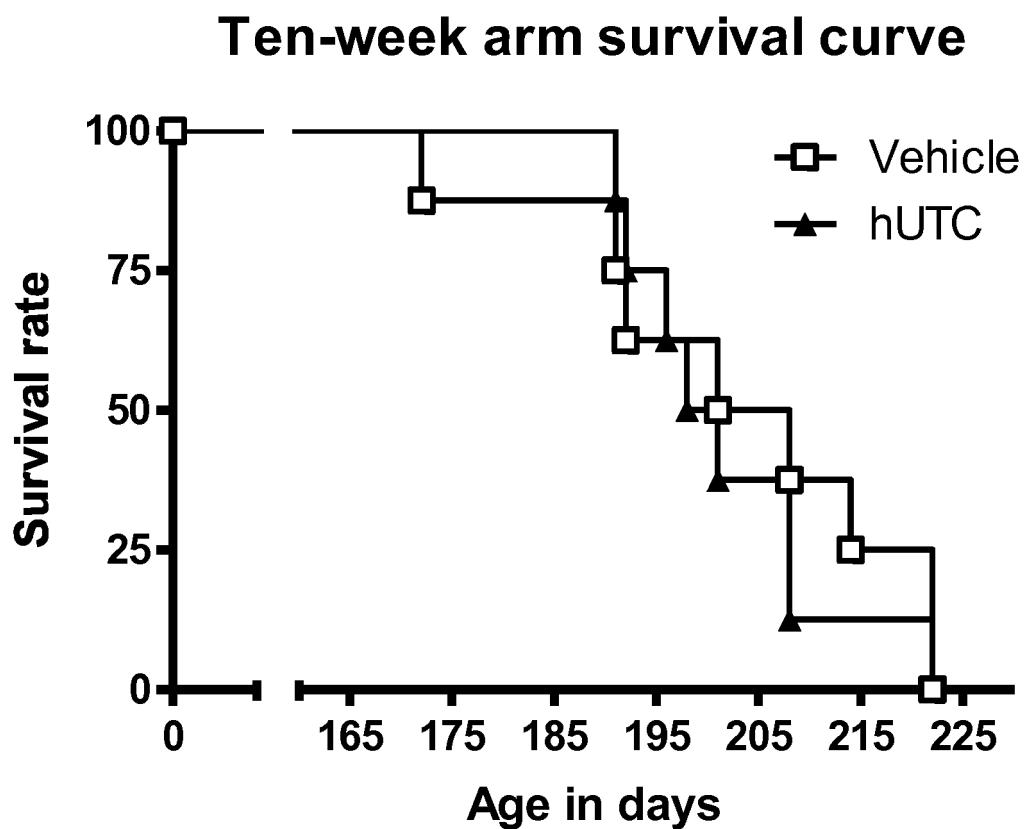


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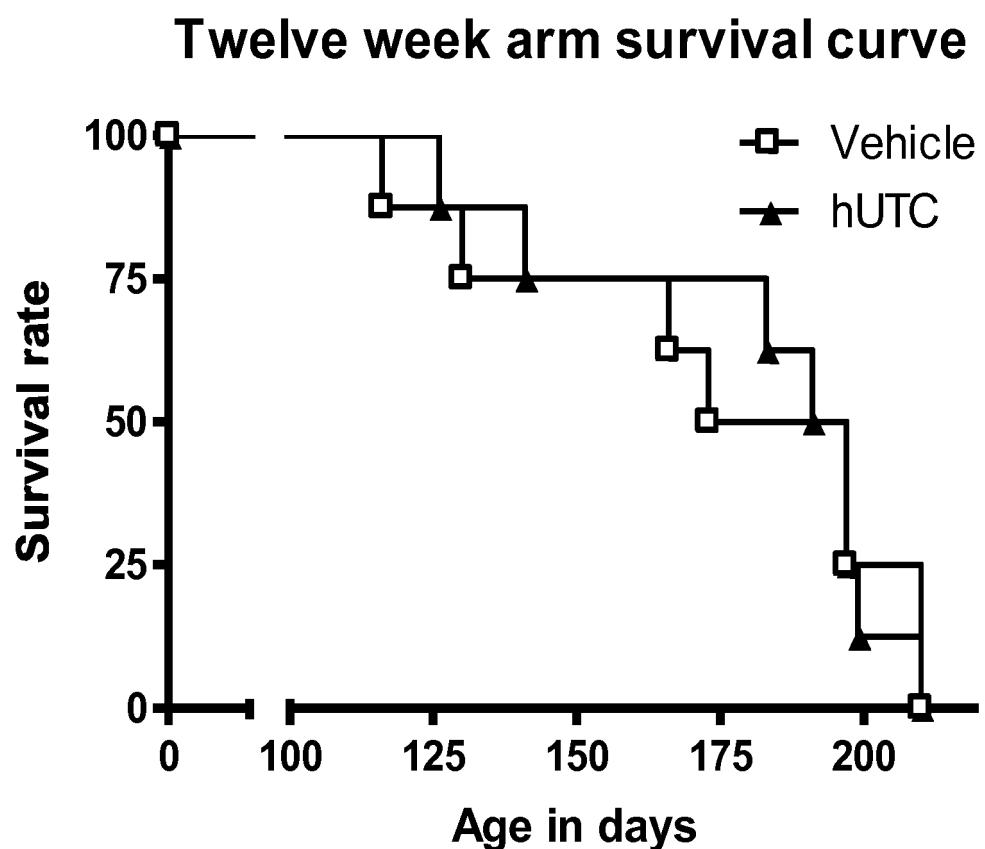


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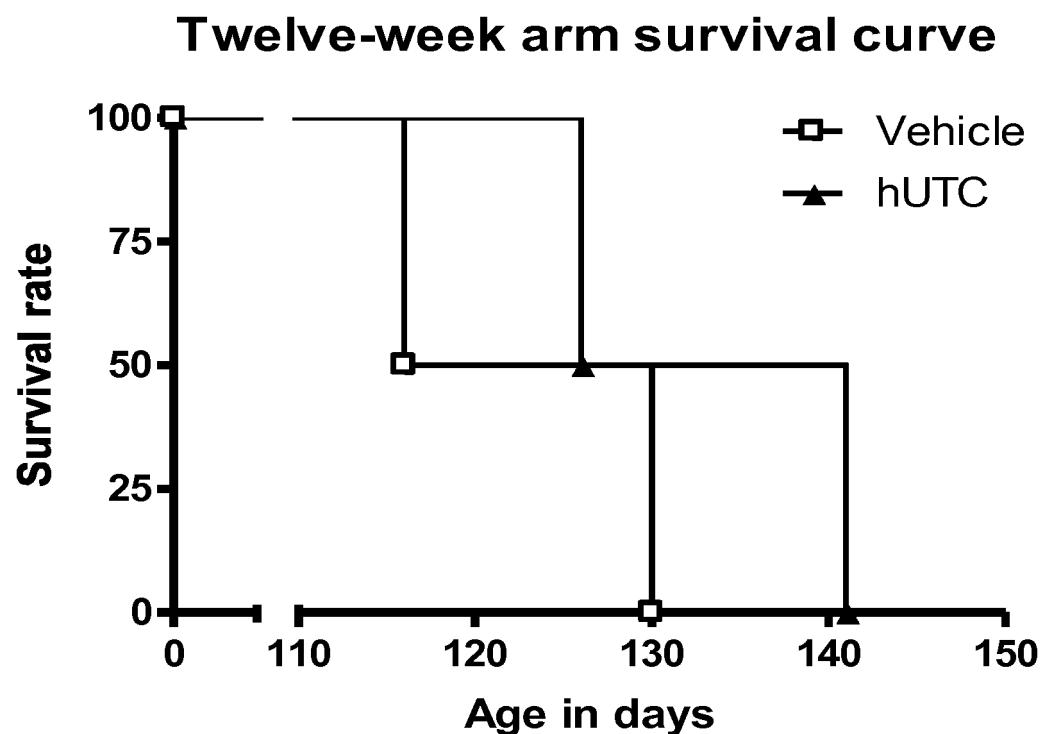


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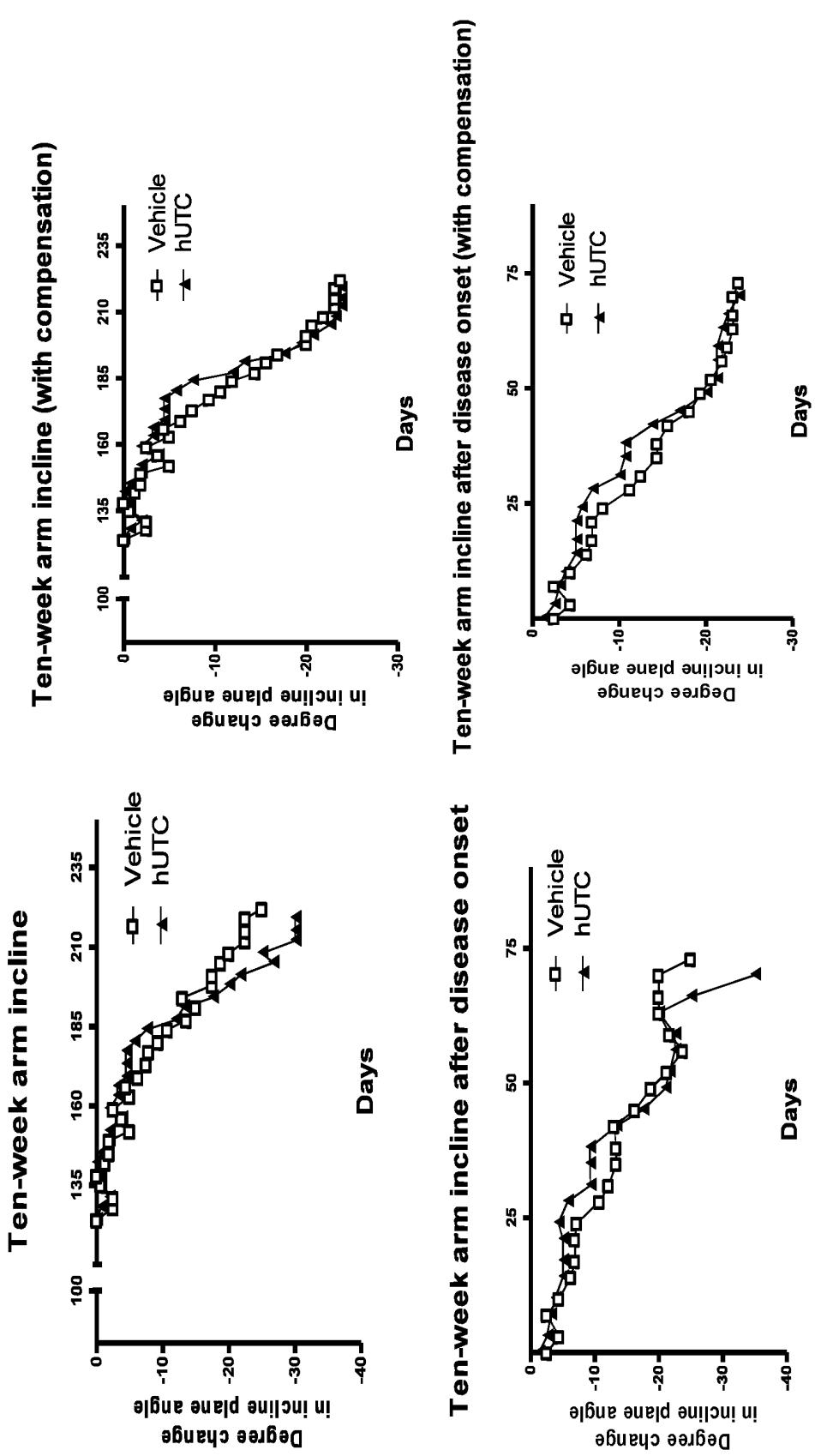
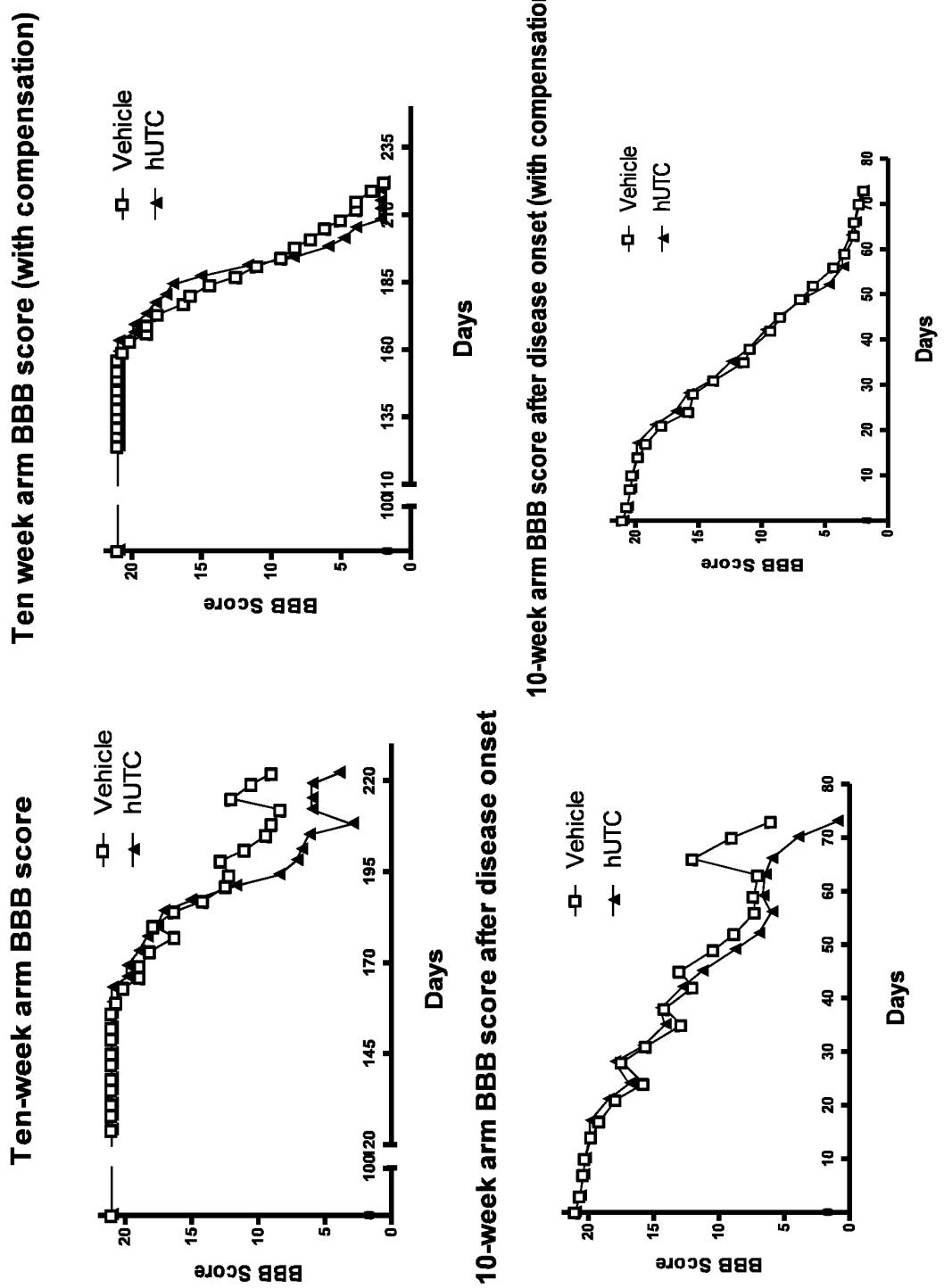


Fig. 13

**Fig. 14**

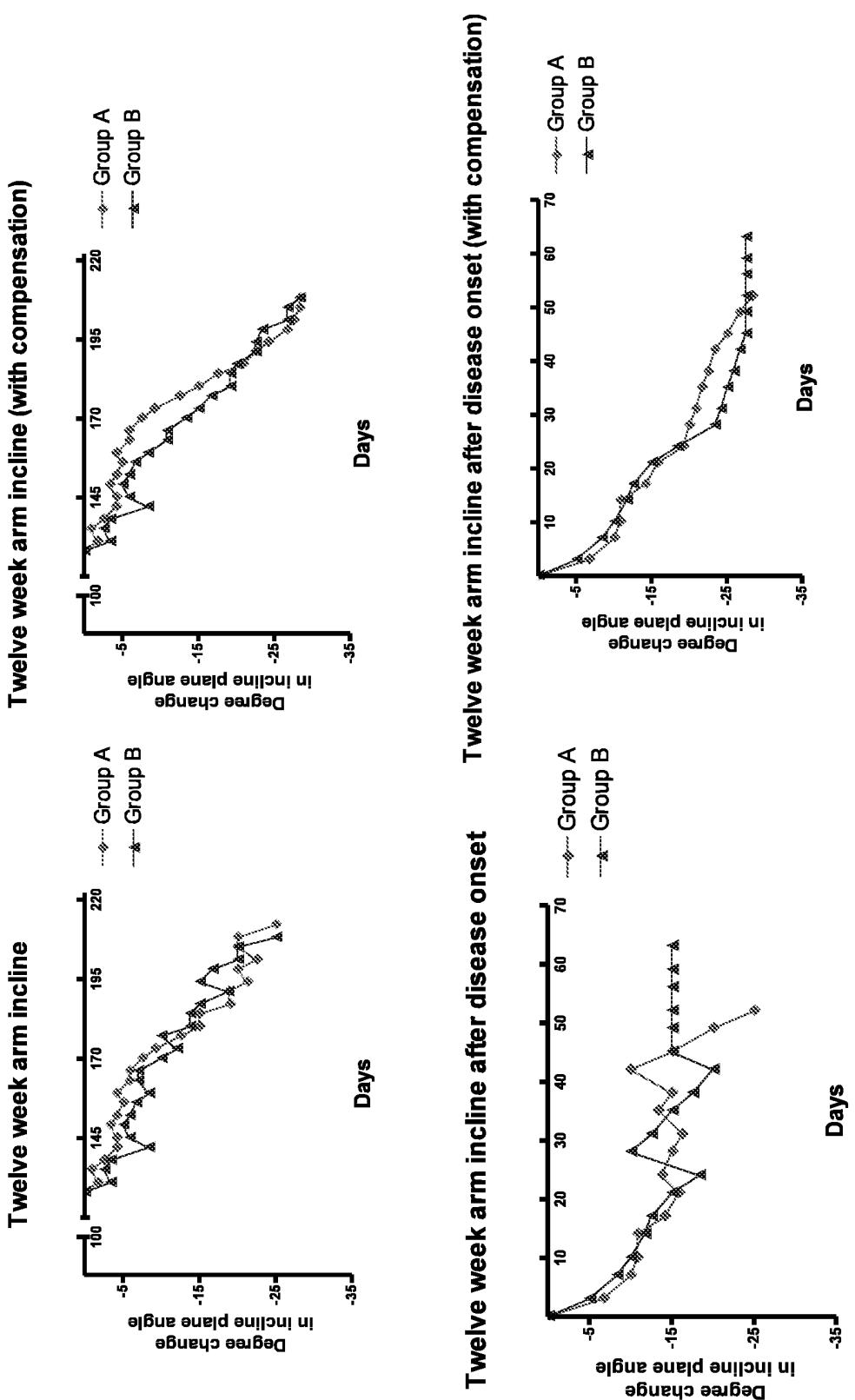


Fig. 15

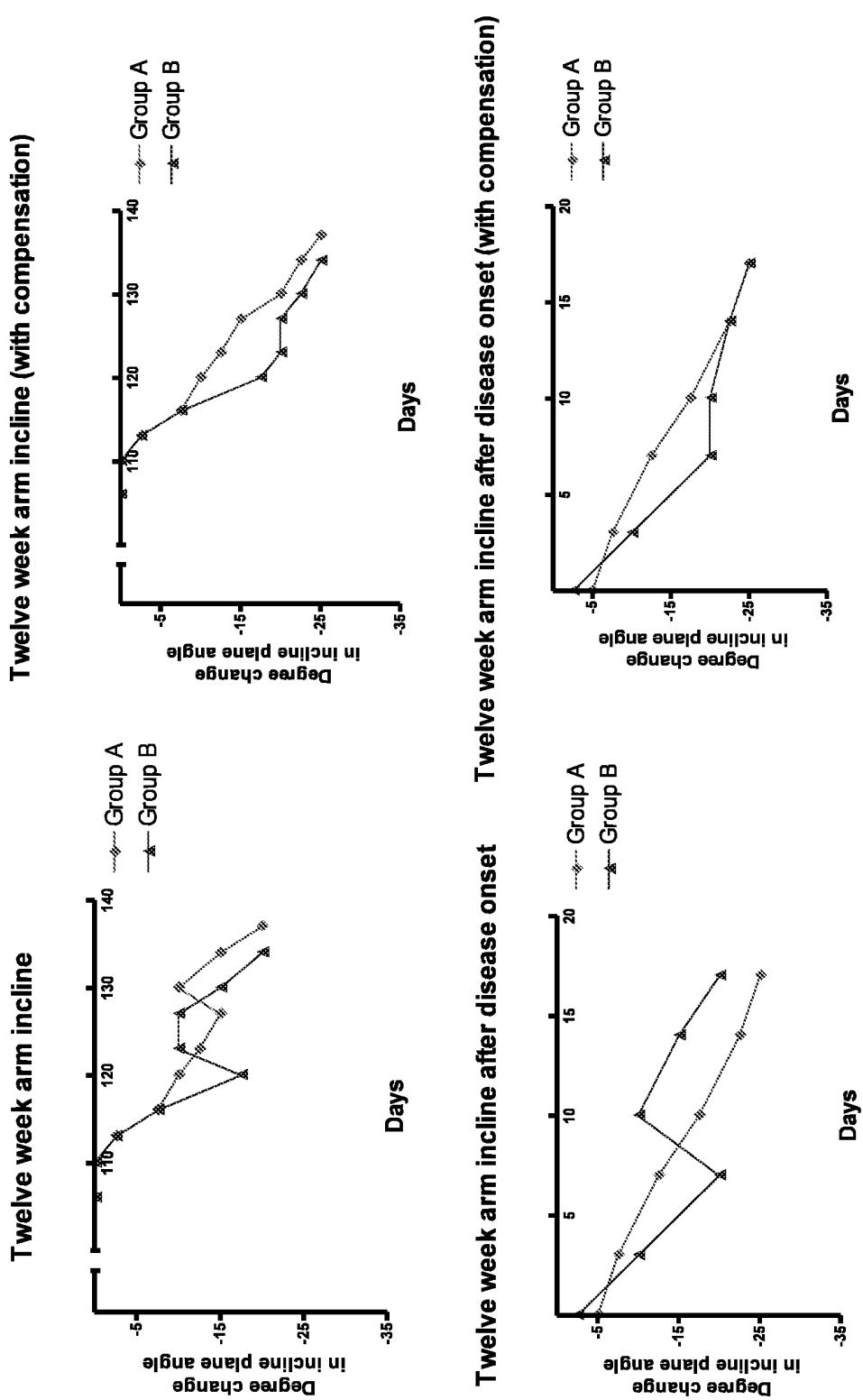


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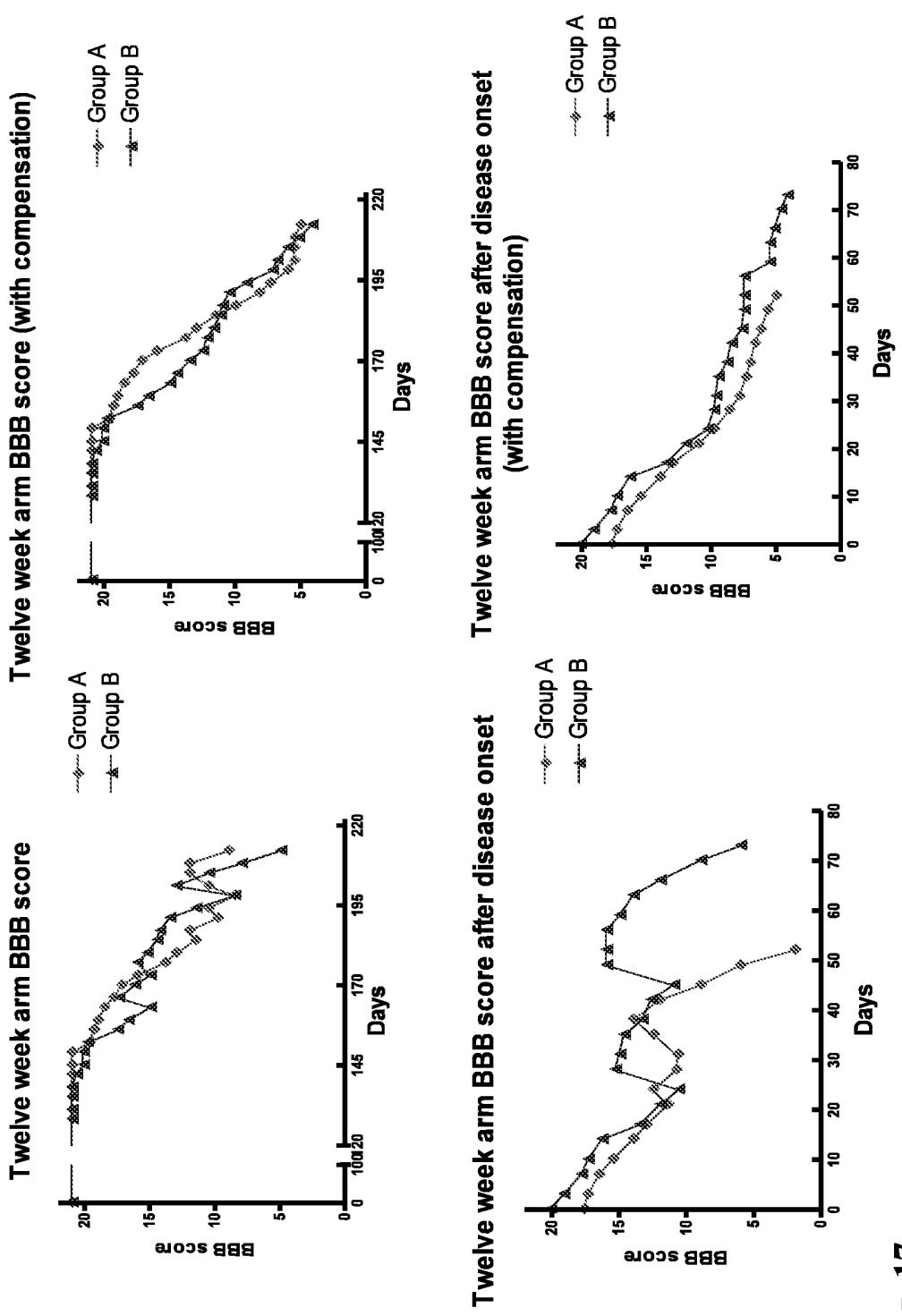


Fig. 17

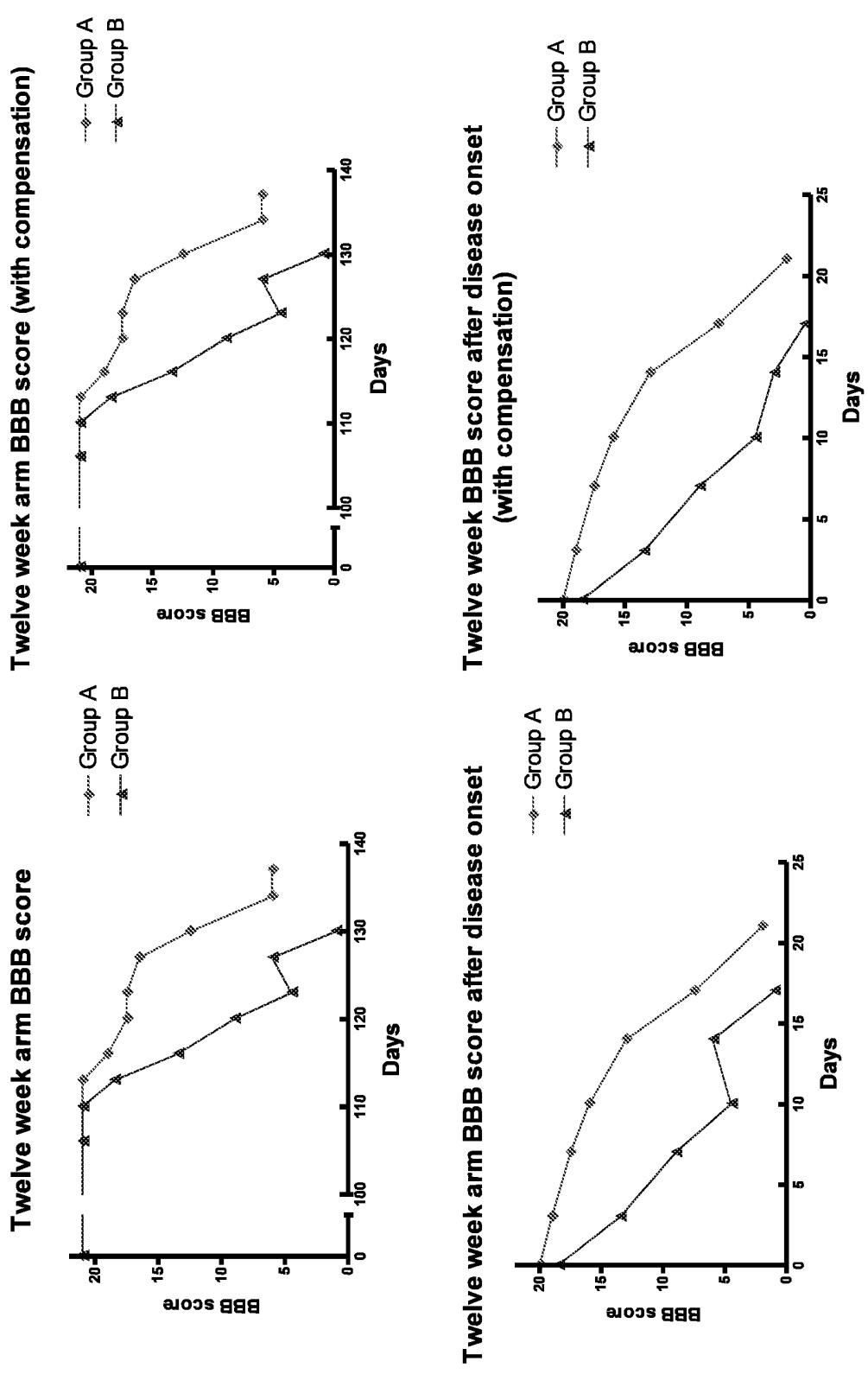
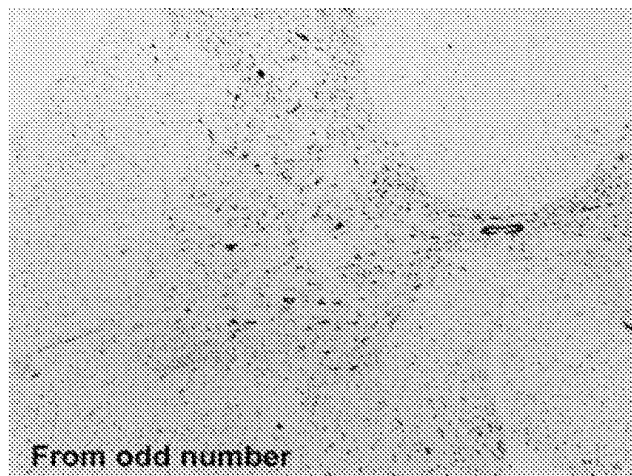
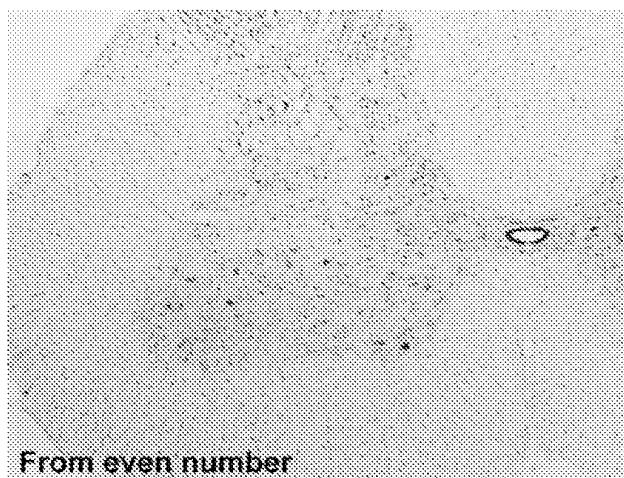


Fig. 18

A.



B.



C.

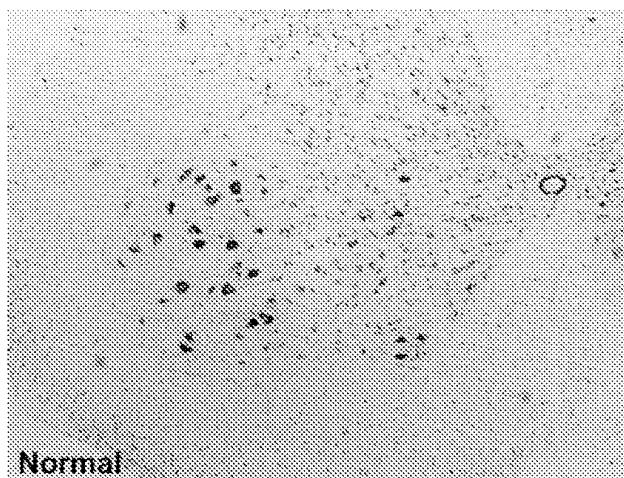


Fig. 19

REFERENCES CITED IN THE DESCRIPTION

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